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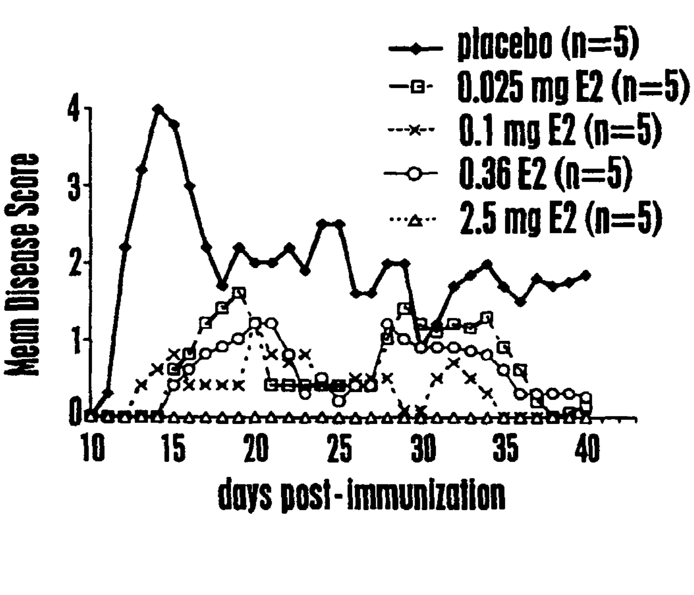
(54) **Title:** METHOD OF TREATING IMMUNE PATHOLOGIES WITH LOW DOSE ESTROGEN



(57) Abstract: The invention provides a method of ameliorating a Th1-mediated immune pathology in a mammal. The method is practiced by administering a low dose of estrogen to the mammal. Optionally, an immunotherapeutic agent can also be administered to the mammal. Also provided are kits containing a low dose of estrogen and an immunotherapeutic agent.

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METHOD OF TREATING IMMUNE PATHOLOGIES WITH LOW DOSE ESTROGEN

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

This invention relates generally to the fields of immunology and medicine and, more specifically, to the use of low dose estrogen to treat immune pathologies.

BACKGROUND INFORMATION

The involvement of female sex hormones in immune pathologies has been proposed based on a number of clinical and experimental observations. First, a variety of autoimmune diseases, including multiple sclerosis, rheumatoid arthritis and Grave's disease, preferentially affect women, and first occur during the reproductive years. Second, during pregnancy, when levels of female sex hormones are high, clinical remissions of cell-mediated autoimmune diseases are common, with disease exacerbation often seen post-partum when sex hormone levels are low. Third, in animal models of autoimmune disease, administration of estrogen at levels equal to or greater than those found in pregnancy has been shown to suppress the clinical and histopathological symptoms of the disease. Fourth, *in vitro*, estrogen at the high concentrations found in pregnancy has been shown to inhibit production of inflammatory cytokines and to stimulate production of anti-inflammatory cytokines by autoantigen-specific CD4+ cells from multiple sclerosis patients. However, in the same study, low concentrations of estrogen had the opposite effect, stimulating production of inflammatory cytokines, with little or no

effect on production of anti-inflammatory cytokines (Correale et al., J. Immunol. 161:3365-3374 (1998).

To explain these observations, it has been proposed that the response to estrogen is biphasic, with high levels associated with protection from autoimmune disease, and low levels associated with promotion of disease. However, because of the potential adverse effects of high levels of estrogen on the reproductive and circulatory systems, and because of the potential unwanted side effects in males, administration of high levels of estrogen is unlikely to be widely useful as a therapy.

The effect of administering low dose estrogen to an individual with an immune disease has not previously been tested, although, from the clinical and experimental observations described above, little or no beneficial effect on the course of the disease would be predicted.

There exists a need to design effective therapies that are applicable for treating a variety of immune pathologies, in both genders, with minimal side effects. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The invention provides a method of ameliorating a Th1-mediated immune pathology in a mammal. The method is practiced by administering a low dose of estrogen to the mammal. Optionally, an immunotherapeutic agent can also be administered to the mammal. Also provided are

kits containing a low dose of estrogen and an immunotherapeutic agent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the effect of various concentrations of 17β-estradiol (E2) on the severity of EAE in female SJL mice.

Figure 2 shows the effect of various concentrations of estriol (E3) on the severity of EAE in female SJL mice.

Figure 3 shows the effect of E2 and E3 on the severity of EAE in male SJL mice.

Figure 4 shows the effect of low dose estrogen therapy on the PLP 139-151 induced proliferation of draining lymph node (DLN) cells from female SJL mice with EAE.

Figure 5 shows the effect of low dose estrogen therapy on PLP 139-151 induced cytokine production by DLN cells from female SJL mice with EAE.

Figure 6 shows the effect of estrogen therapy on PLP 139-151 specific immunoglobulin production in female SJL mice with EAE.

Figure 7 shows the effect of administration of BV8S2 (Vβ8.2) protein in IFA on the development of EAE in male and female mice.

Figure 8 shows the effect of 17β-estradiol and estriol on cumulative EAE disease index in intact and ovariectomized female mice.

Figure 9 shows the effect of administration of BV8S2 protein, 17β-estrdiol and the combination of BV8S2 vaccination plus estrus levels of 17β-estradiol on EAE in Tg females.

Figure 10 shows T cell proliferative (A-C) and antibody (D) responses in BV8S2 and/or E2 treated and control Tg mice challenged to develop EAE.

Figure 11 shows the effect of ovariectomy (OVX) and treatment with estrus levels of 17β-estradiol (E2) on the course of EAE in Tg female mice.

Figure 12 shows data obtained from RPA analysis of chemokine mRNA expression in spinal cords of intact and ovariectomized 17β-estradiol treated and control TCR BV8S2 transgenic female mice.

Figure 13 shows data obtained from RPA analysis of chemokine expression in spinal cord (SC) tissue and mononuclear cells from SC of BV8S2 transgenic mice with EAE.

Figure 14 shows data obtained from RPA analysis of chemokine receptor expression in SC of 17β-estradiol treated, ovariectomized and control TCR BV8S2 transgenic female mice with EAE.

Figure 15 shows data obtained from RPA analysis of cytokine mRNA expression in SC of 17β-estradiol treated and control TCR BV8S2 transgenic female mice with EAE.

Figure 16 shows the effect of 17β-estradiol on *in vitro* proliferative (A) and cytokine (B) responses of lymphokine (LN) T cells from naïve TCR BV8S2 transgenic female mice.

Figure 17 shows the effect of 17β-estrodiol on the severity of EAE in wildtype C57BL/6 and cytokine knockout mice.

Figure 18 shows data obtained from RPA analysis of chemokine and chemokine receptor mRNA expression in the spinal cords of untreated and estrogen treated wildtype and cytokine knockout mice with EAE.

Figure 19 shows cytokine production in the CNS of untreated and estrogen treated wildtype and cytokine knockout mice. Panel A shows data obtained from RPA analysis of total RNA from the spinal cords of mice at the peak of EAE. Panel B shows by FACS analysis the percentages of Vβ8.2+, MOG 35-55 stimulated T cells that express the indicated cytokines with or without 17β-estradiol treatment.

Figure 20 shows the effect of estrogen treatment on MOG 35-55 stimulated T cell proliferation and the expression of cell adhesion and activation markers in wildtype and cytokine knockout mice.

Figure 21 shows the effect of estrogen treatment on the frequency of Vβ8.2 T cells expressing the indicated cytokines.

DETAILED DESCRIPTION OF THE INVENTION

As disclosed herein, administration of a low dose of estrogen unexpectedly reduces the severity of Th1-mediated immune pathologies. Additionally, low dose estrogen and immunotherapeutic agents act synergistically in reducing the severity of Th1-mediated immune pathologies. These effects appear to be due, in part, to the effect of low dose estrogen on reducing the expression of pro-inflammatory cytokines and chemokines by T cells in the periphery and at the site of the pathology.

Therefore, the invention provides a novel method of preventing or ameliorating immune pathologies in a mammal by administering to the mammal a low dose of estrogen. Optionally, the method further comprises administering an immunotherapeutic agent. The methods are advantageous in that low doses of estrogen can be administered to both males and females to prevent or ameliorate immune pathologies. Side effects of high dose estrogen therapy, which are detrimental in females and preclude its use in males, are expected to significantly reduced by administering low dose estrogen. Additionally, the synergistic effects of low-dose estrogen and an immunotherapeutic agent in inhibiting pathogenic immune responses allow a lower dose of agent to be used than required using the agent alone, which reduces potential side effects and lowers the cost of therapy.

The methods of the invention can be practiced with respect to a variety of immune pathologies. As used herein, the term "immune pathology" refers to a pathology mediated by a detrimental immune response. Such pathologies are known in the art and include, but are not limited to, autoimmune pathologies, immune reactions by or against allografts, immune responses to infectious agents, chronic immune responses, allergic reactions and immunosuppressive responses and immunoproliferative pathologies. Prognostic indicators and clinical signs associated with particular immune pathologies are well known in the art. Additionally, as described below, immunotherapeutic agents useful in preventing or treating immune pathologies are well known in the art, which can effectively be used in combination with low dose estrogen therapy in the methods of the invention.

Those skilled in the art appreciate that many immune pathologies are mediated by a combination of Th1, Th2, antibody, B cell, phagocytic and complement responses. Preferably, the methods of the invention are practiced with respect to "Th1-mediated pathologies," which are pathologies in which the detrimental immune response is primarily or partially a T helper 1 (Th1) type immune response. A Th1 immune response is characterized by secretion of pro-inflammatory cytokines, which include IL-12, IFN-γ and TNF-α. Th1-mediated pathologies include most autoimmune diseases, many alloimmune disorders, certain allergic conditions, certain chronic inflammatory conditions, and certain infectious conditions.

An immune pathology can alternatively be mediated by a "Th2 immune response," which indicates that the detrimental immune response is partially or primarily

of the T helper 2 (Th2) type. A Th2 response is characterized by secretion of anti-inflammatory cytokines, such as IL-4, IL-10, IL-13 and TGF-β.

In most autoimmune pathologies, T cells recognize a host component in one or more tissues as foreign, and attack that tissue. Exemplary autoimmune pathologies affecting mammals include rheumatoid arthritis (RA), juvenile oligoarthritis, collagen-induced arthritis, Sjogren's syndrome, multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE), inflammatory bowel disease (e.g. Crohn's disease, ulceritive colitis), autoimmune gastric atrophy, pemphigus vulgaris, psoriasis, vitiligo, type I diabetes, myasthenia gravis, Grave's disease, Hashimoto's thyroiditis, sclerosing cholangitis, sclerosing sialadenitis, systemic lupus erythematosis, Addison's disease, systemic sclerosis, polymyositis, dermatomyositis, pernicious anemia, and the like.

Alloimmune pathologies occur when tissue is transplanted from a donor whose HLA antigens do not completely match the recipient antigens. The donor cells can be recognized by the recipient immune system as foreign, resulting in rejection of the transplanted tissue. Alternatively, donor immune cells can recognize the recipient tissues as foreign, and attack the recipient (graft versus host disease). Thus, a pathogenic alloimmune response can be a response by or against a transplanted organ (e.g. heart, blood vessel, valve, liver, lung, kidney, skin) or infused hematopoietic cells, such as an apheresis product or bone marrow.

Septic shock is a life-threatening response to infectious agents. High levels of bacterial toxins, including exotoxins and endotoxins, can inappropriately activate the host immune system to produce hypersensitivity reactions that rapidly leading to septic shock with associated organ failure and death. Septic shock is associated with both gram-negative bacteria, such as *Staphylococcus* species, *Streptococcus* species, as well as gram-positive bacteria, and often is associated with infection following surgery or trauma.

Chronic inflammatory responses are often initially responses to infectious agents, but can be of any etiology, including tissue trauma. Chronic inflammatory responses are associated with a variety of diseases, including cardiovascular disease, coronary disease, cirrhosis, arthritis, cholestasis, tuberculosis, leprosy, syphilis, periodontitis, fibrosis, glomerulonephritis, and certain cancers.

Infectious agents that cause chronic inflammatory immune responses include, for example, bacteria (e.g. *Helicobacteria; Mycobacteria; Spirochocae; Yersinia* and the like), viruses (e.g. HIV, hepatitis viruses, herpes simplex viruses, papovavirus, rabies virus), fungi, protozoa, helminths and prions.

Allergic reactions are hypersensitivity reactions to agents in the environment. Allergic reactions can be IgE/mast cell mediated, antibody mediated, Th1 or Th2 cell mediated, or a combination thereof. Allergic conditions and their etiology are well known in the art. Common allergic conditions include, for example, asthma, hay fever and food allergies.

Other immune pathologies that can be amenable to treatment with low dose estrogen include immune deficiency disorders, wherein a mammal mounts an inadequate immune response. Immune deficiencies can be caused, for example, by HIV, the causative agent of AIDS; by malignancy; by old age; by malnutrition; by metabolic disease; by drug therapy; or by splenectomy. Immune pathologies that can be amenable to treatment with low dose estrogen include further include T cell replicative pathologies, including T cell leukemias and lymphomas.

Low dose estrogen therapy, alone or in combination with immunotherapeutic agents or conventional therapies (e.g. antibiotics, antiviral agents, chemotherapy, radiation, as appropriate for the particular disease), can be used to reduce the severity of the immune pathologies described above. As described herein, because of the inhibitory effect of low dose estrogen on TNFα expression, chemokine expression and chemokine receptor expression, low dose estrogen therapy will be particularly useful in pathologies mediated by Th1 type inflammatory responses.

As used herein, the term "ameliorating," with reference to an immune pathology, refers to any observable beneficial effect of the treatment. The beneficial effect can be evidenced, for example, by a delayed onset of clinical symptoms in a susceptible mammal, a reduction in severity of some or all clinical symptoms of the disease, a slower progression of the disease, a reduction in the number of relapses of the disease, a reduction in the number or activity (e.g. Th1 type cytokine secretion) of pathogenic T cells at the site of pathology or in the circulation, an improvement in the overall health or well-being of the individual, or

by other parameters well known in the art that are specific to the particular disease. Those skilled in the art can determine, based on knowledge of the expected course of the particular disease, whether there is a delayed onset of clinical symptoms. Those skilled in the art can also determine whether there is an amelioration of the clinical symptoms or reduction in the number or activity of pathogenic T cells following treatment as compared with before treatment or as compared to an untreated mammal.

A useful method of monitoring the effect of a treatment that potentially ameliorates multiple sclerosis is magnetic resonance imaging, or MRI. As used herein, the term "magnetic resonance imaging" refers to conventional MRI methods, as well as improved magnetic resonance (MR) techniques, such as cell-specific imaging, magnetization transfer imaging (MTI), gadolinium (Gd)-enhanced MRI, proton magnetic resonance spectroscopy (MRS), diffusion-weighted imaging (DWI), functional MR imaging (fMRI), and the other neuro-imaging methods known in the art. MRI methods and their applications to MS are described, for example, in Rovaris et al, <u>J. Neurol. Sci</u>. 186 Suppl 1:S3-9 (2001). MRI techniques allow an assessment of the effects of treatment on amelioration of a variety of well-known indicia of MS, including edema, blood brain barrier break-down, demyelinisation, gliosis, cellular infiltration, axonal loss, T2 lesion load, T1 lesion load, gadolinium positive lesion load, and the like.

As used herein, the term "mammal" refers to a human, a non-human primate, canine, feline, bovine, ovine, porcine, murine or other veterinary or laboratory mammal. Those skilled in the art understand that the

immune responses and immune pathologies of mammals share many common features, and that a therapy which reduces the severity of an immune pathology in one species of mammal is predictive of the effect of the therapy on another species of mammal. The skilled person also appreciates that credible animal models of many human immune pathologies are known. As described in the Example, EAE is a credible animal model of human multiple sclerosis.

As used herein, the term "estrogen" refers to the steroids commonly known as 17β-estradiol (E2), estrone (E1) and estriol (E3). Also included within the term "estrogen" are metabolites and derivatives of E1, E2 and E3. Such metabolites and derivatives act as agonists of the estrogen receptor (ERα or ERβ) and have a similar core steroid structure as E1, E2 or E3, but can have one or more different groups (e.g. hydroxyl, ketone, halide, etc.) at one or more ring positions. Those skilled in the art can readily determine whether such metabolites and derivatives are agonists of estrogen by *in vitro* assays that measure signaling through the estrogen receptor. Alternatively, the effects of metabolites and derivatives of estrogen can be assessed, and compared to the effects of known estrogens, using any of the *in vivo* and *in vitro* assays that report estrogen's effects, as described in the Examples, below.

The methods of the invention can also be practiced with a non-steroidal estrogen analog that acts as an agonist of the estrogen receptor. Methods of identifying receptor agonists from libraries of compounds are well known in the art, and include binding assays (e.g. competitive and non-competitive radioimmunoassays) and signaling assays (e.g. transcription-based assays

using reporter genes driven by an estrogen response element). Libraries of naturally occurring and synthetic compounds, including inorganic compounds, peptides, lipids, saccharides, nucleic acids and small organic molecules, are commercially available, and can be screened in high-throughput assays to identify estrogen analogs.

In the methods of the invention, estrogen is administered at a low but sufficient dose to reduce the severity of the particular immune pathology exhibited by the mammal. The dose will depend, among other considerations, on the type of estrogen, its formulation and route of administration, the duration of therapy, the type and severity of the pathology, and on the weight and gender of the mammal.

As used herein, the term "low dose" refers to an amount sufficient to raise the serum concentration above basal levels, but below pregnancy levels. The diestrus, estrus and pregnancy serum concentrations of E2 and E3 in mice are shown in Table 1. Human female physiologic concentrations of E1 and E3 are roughly equivalent to those of E2, which circulates at 10 to 1,000 pg/ml during the normal menstrual cycle, and up to 35,000 pg/ml during pregnancy.

TABLE 1

	17β-estradiol (pg/ml)	**Estriol (pg/ml)**
Diestrus	20-30	<100
Estrus	100-200	<100
Pregnancy	5,000-10,000	2,000-3,000

Thus, a low dose of estrogen can raise serum E1, E2 or E3 to at least 10 pg/ml, such as 20 pg/ml, 30 pg/ml, 40 pg/ml, 50 pg/ml, 75 pg/ml, 100 pg/ml, 150 pg/ml, 200 pg/ml, 300 pg/ml, 400 pg/ml, 500 pg/ml, 750 pg/ml, 1000 pg/ml, 1500 pg/ml, and generally will not raise serum E1, E2 or E3 beyond 2000 pg/ml. The amount of estrogen to administer to achieve desired hormone levels in the serum is known in the art, and will depend, for example, on the weight of the mammal, the half-life of the particular estrogen, and the route and form of administration. The efficacy of a particular dose of estrogen can be monitored and adjusted during therapy by examining standard disease parameters.

Those skilled in the art can determine an appropriate time and duration of therapy to achieve the desired preventative or ameliorative effects on the immune pathology. Thus, the methods of the invention can be practiced so as to maintain low levels of estrogen in the blood for several days, weeks, months or years, or over the course of the lifetime of the individual. For example, the therapy can be administered continuously to an individual at risk of developing an immune pathology, such as an individual with a genetic predisposition to a pathology, or with preclinical indications of the pathology. Likewise, estrogen can be administered continuously to an individual early or late in the course of the disease, or only administered during exacerbations of the disease until symptoms are controlled.

Low doses of estrogen can be prepared in any convenient form and administered by any convenient route known in the art. Preferably, for human therapy, estrogen will be administered orally, transdermally, subcutaneously, intravenously, intramuscularly, by a

respiratory route (e.g. inhalation), intranasal, enteral, topical, sublingual, or rectal means. Estrogen can also be administered directly to the site of the pathology, such as skin lesions, inflamed joints, into the central nervous system, and the like. For continuous release of defined concentrations of estrogen, administration via micropumps, biopolymers, liposomes and other slow-release vehicles is advantageous.

Optionally, low dose estrogen therapy can be combined with administration of an immunotherapeutic agent. As used herein, the term "immunotherapeutic agent" refers to any compound used prophylactically or therapeutically to inhibit an immune response or to ameliorate an immune pathology. Preferably, the immunotherapeutic agent will be administered at a lower dose than that required for complete efficacy on its own, such that when combined with administration of a low dose of estrogen, there will be a pronounced effect on reduction of disease severity not achieved by the immunotherapy alone. Administering a lower dose of immunotherapeutic agent reduces the risk of adverse effects, as well as reduces the cost of therapy.

The immunotherapeutic agent can be administered in combination with estrogen or separately; either before, at the same time, or after estrogen administration; either by the same route or by a different route (e.g. any of the routes described above); and either at the same site or at a different site. Those skilled in the art can determine appropriate conditions for administering both a low dose of estrogen and an immunotherapeutic agent to a mammal.

The choice of immunotherapeutic agent to use and route and site of administration will depend on the particular immune pathology. A variety of agents with at least partial efficacy in treating immune pathologies are known in the art, and their mechanisms of action are often well understood. Other immunotherapeutic agents with similar mechanisms of action are in development.

The activation of a T cell immune response requires interaction between a T cell receptor on the surface of the pathogenic T cell, and an antigenic peptide bound to an HLA (MHC) molecule on the surface of an antigen presenting cell or target cell. Any agent which disrupts this trimolecular complex can be effective in combination with low dose estrogen therapy in reducing the T cell immune response. Agents which disrupt the trimolecular complex can be either immunomodulatory agents or immunoblocking agents, or act by both an immunomodulatory and a blocking mechanism.

As used herein, the term "immunomodulatory agent" is intended to refer to an agent that induces a host immune response, such as a tolerogenic response or an active immune response in a mammal.

Exemplary immunomodulatory agents that cause a tolerogenic response, which leads to immunological unresponsiveness, are autoantigens targeted by pathogenic T cells in an autoimmune response. Autoantigens and the adminstration of these autoantigens by a variety of routes (including oral and intravenous routes) so as to induce tolerance are known in the art and described, for example, in U.S. Patent Nos. 6,039,947; 6,019,971; 5,869,093; 5,858,968 and 5,856,446.

Known or suspected autoantigens, with their associated diseases, include: myelin basic protein, proteolipid protein, major oligodendrocytic protein, myelin associated glycoprotein, and αB-crystallin (multiple sclerosis and EAE); collagen type II, heat shock proteins, aggrecans, proteoglycans, fillagrin and link (collagen-induced arthritis, adjuvant-induced arthritis, rheumatoid arthritis); desmin (psoriasis); S-antigen (uveitis); insulin, glutamic acid decarboxylase (NOD, type I diabetes); tropomyosin (inflammatory bowel disease); epidermal cadherin (pemphigus vulgaris); Sm, RNP, histones (systemic lupus erythematosus); thryoid stimulating hormone receptor (Grave's disease); thyroglobulin, peroxidase (Hashimoto's thyroiditis); collagen type IV (Goodpasture's syndrome); platelet integrin a IIb: IIIa (autoimmune thrombocytopenia purpura); Rh blood group 1 antigen (autoimmune hemolytic anemia); and acetylcholine receptor (myasthenia gravis). For allotransplation, allopeptides or allogeneic T cells can serve as tolerogens.

Immunomodulatory agents that induce an active immune response include vaccines that elicit an immune response that specifically or non-specifically targets pathogenic T cells. Non-specific vaccines include, for example, vaccines containing antigens present on all or most T cells (e.g. CD2, CD3, CD4, CD5, CD6, CD7, CD8, CD27, CD28, CD32, CD43, and T cell receptor constant regions).

T cells express on their surface a heterodimeric T cell receptor, composed of either α/β chains or γ/δ chains. There are now known to be at least 24 β chain variable region gene families, some of which have multiple family members, and also a large number of α

chain variable region gene families. It is well established that many autoimmune and infectious pathologies are mediated by T cells expressing a limited repertoire of T cell receptors, which are clonally expanded in response to antigen or superantigen stimulation. For example, EAE has been demonstrated to be associated with rodent Vβ8.2 (BV8S2) expressing T cells; psoriasis with human Vβ3, Vβ13.1 and Vβ17 expressing T cells; diabetes with human Vβ6.1, Vβ6.6/6.7 and Vβ14 expressing T cells; multiple sclerosis with Vβ2, Vβ5 (e.g. Vβ5.1 and Vβ5.2), Vβ6 (e.g. Vβ6.1, Vβ6.2, Vβ6.5, Vβ6.7), Vβ7 and Vβ13 expressing T cells; and rheumatoid arthritis and certain superantigen-mediated infectious diseases with Vβ3, Vβ14 and Vβ17 expressing T cells. Clonally expanded populations of T cells are also associated with T cell proliferative pathologies, such as T cell leukemia and T cell lymphoma.

Those skilled in the art can determine the T cell receptor or receptors present on the relevant pathogenic T cells for the particular pathology or particular individual using methods described, for example, in U.S. Patents 5,612,035; 5,861,164; 6,007,815; 5,837,246; 5,985,552; 5,614,192; 5,223,426; 6,113,903, and 5,776,459, and in PCT publications 95/21623; 93/06135; 94/25063; 99/27957 and 95/00658. Briefly, as described in these references, T cell receptor usage by the relevant T cells (such as CD25+ activated T cells, antigen-responsive T cells, or T cells from the site of the pathology) can be determined by the polymerase chain reaction using a panel of V-region specific primers, or by using anti-TCR antibodies.

Administration of T cell clones expressing T cell receptors associated with the particular pathology, or administration of the corresponding intact dimeric T cell receptors, full-length single T cell receptor chains or portions thereof, variable region peptides or portions thereof, or complementarity determining region peptides (e.g. the CDR1, CDR2, CDR3 or CDR4 region) or portions thereof, and analogs of these sequences, alone or in combination, have been shown to induce immune responses specific for the pathogenic T cells that reduce the activity or number of pathogenic T cells. Thus, such molecules can be used as immunomodulatory agents in combination with low dose estrogen administration to reduce the severity of immune pathologies.

The sequences of T cell receptor α, β, γ or δ variable and constant regions from a variety of species are well known in the art (see, for example, Genevee et al., Eur J Immunol. 22:1261-1269 (1992); Arden et al., Immunogenetics 42:455-500 (1995); Choi et al., Proc. Natl. Acad. Sci. USA 86:8941-8945 (1989); Concannon et al., Proc. Natl. Acad. Sci. USA 83:6598-6602 (1986); Kimura et al., Eur J Immunol. 17:375-383. (1987); Robinson, J. Immunol. 146: 4392-4397 (1991); and the EMBL alignment database under alignment accession number DS23485)). Methods of preparing and administering T cell receptors, single chains, and characteristic peptides therefrom, so as to stimulate an immune response specific for the corresponding pathogenic T cell, are described, for example, in Example II, below, as well as in the patents and PCT publications referenced above. Single chain peptides can contain, for example, from about 8 to about 100 amino acids, such as from about 10 to about 50 amino acids, including from about 15 to about 30 amino acids.

Advantageously, T cell receptor heterodimers, individual T cell receptor chains, T cell receptor variable regions, and fragments from any of these molecules can contain or consist of amino acid sequences from the CDR2 hypervariable region. The location of the CDR2 region for each variable chain is known in the art, and is generally at about amino acid residues 38-58 of most human Vβs and Vαs. Exemplary TCR peptides that include some or all of a Vβ (BV) or Vα (VA) CDR2 region are shown in Table 2:

TABLE 2

TCR	Amino Acid Sequence	SEQ ID NO:
Vβ3	LGLRLIYFSYDVKMKEKGDI	1
Vβ5.2	ALGQGPQFIFQYYEEEERQRG	2
Vβ5.2 (Y49T)	ALGQGPQFIFQTYEEEERQRG	3
Vβ6.1	LGQGPEFLIYFQGTGAADDSG	4
Vβ6.5	LGQGPEFLTYFQNEAQLEKS	5
Vβ13	GLRLIHYSVGAGITDQGEV	6
Vβ14	SMNVEVTDKGDVPEGYK	7
Vβ17	SQIVNDFQKGDIAEGYS	8
BV1S1A1N1	SLDQGLQFLIQYYNGEERAKG	9
BV1S1A2	SLDQGLQFLIHYYNGEERAKG	10
BV2S1A1	FPKQSLMLMATSNEGSKATYE	11
BV2S1A3N1	FPKKSLMLMATSNEGSKATYE	12
BV2S1A4T	FPKQSLMLMATSNEGCKATYE	13
BV2S1A5T	FPKKSLMQIATSNEGSKATYE	14
BV3S1	DPGLGLRLIYFSYDVKMKEKG	15
BV4S1A1T	QPGQSLTLIATANQGSEATYE	16
BV5S1A1T	TPGQGLQFLFEYFSETQRNKG	17
BV5S1A2T	TLGQGLQFLFEYFSETQRNKG	18
BV5S2	ALGQGPQFIFQYYEEEERQRG	19

BV5S3A1T	VLGQGPQFIFQYYEKEERGRG	20
BV5S4A1T	ALGLGLQLLLWYDEGEERNRG	21
BV5S4A2T	ALGLGLQFLLWYDEGEERNRG	22
BV5S6A1T	ALGQGPQFIFQYYREEENGRG	23
BV6S1A1N1	SLGQGPEFLIYFQGTGAADDS	24
BV6S1A3T	SLGQGPELLIYFQGTGAADDS	25
BV6S2A1N1T	ALGQGPEFLTYFQNEAQLDKS	26
BV6S3A1N1	ALGQGPEFLTYFNYEAQQDKS	27
BV6S4A1	TLGQGPEFLTYFQNEAQLEKS	28
BV6S4A4T	NPGQGPEFLTYFQNEAQLEKS	29
BV6S5A1N1	SLGQGLEFLIYFQGNSAPDKS	30
BV6S6A1T	ALGQGPEFLTYFNYEAQPDKS	31
BV6S8A2T	TLGQGSEVLTYSQSDAQRDKS	32
BV7S1A1N1T	KAKKPPELMFVYSYEKLSINE	33
BV7S2A1N1T	SAKKPLELMFVYSLEERVENN	34
BV7S3A1T	SAKKPLELMFVYNFKEQTENN	35
BV8S1	TMMRGLELLIYFNNNVPIDDS	36
BV8S3	TMMQGLELLAYFRNRAPLDDS	37
BV9S1A1T	DSKKFLKIMFSYNNKELIINE	38
BV10S1P	KLEEELKFLVYFQNEELIQKA	39
BV10S2O	TLEEELKFFIYFQNEEIIQKA	40
BV11S1A1T	DPGMELHLIHYSYGVNSTEKG	41
BV12S1A1N1	DPGHGLRLIHYSYGVKDTDKG	42
BV12S2A1T	DLGHGLRLIHYSYGVQDTNKG	43
BV12S2A2T	DLGHGLRLIHYSYGVKDTNKG	44
BV12S2A3T	DLGHGLRLIHYSYGVHDTNKG	45
BV12S3	DLGHGLRLIYYSAAADITDKG	46
BV13S1	DPGMGLRLIHYSVGAGITDQG	47
BV13S2A1T	DPGMGLRLIHYSVGEGTTAKG	48
BV13S3	DPGMGLRLIYYSASEGTTDKG	49
BV13S4	DPGMGLRRIHYSVAAGITDKG	50
BV13S5	DLGLGLRLIHYSNTAGTTGKG	51
BV13S6A1N1T	DPGMGLKLIYYSVGAGITDKG	52
BV13S7	DPGMGLRLIYYSAAAGTTDKE	53

BV14S1	DPGLGLRQIYYSMNVEVTDKG	54
BV15S1	DPGLGLRLIYYSFDVKDINKG	55
BV16S1A1N1	VMGKEIKFLLHFVKESKQDES	56
BV17S1A1T	DPGQGLRLIYYSQIVNDFQKG	57
BV17S1A2T	DPGQGLRLIYYSHIVNDFQKG	58
BV18S1	LPEEGLKFMVYLQKENIIDES	59
BV19S1P	NQNKEFMLLISFQNEQVLQET	60
BV19S2O	NQNKEFMFLISFQNEQVLQEM	61
BV20S1A1N1	AAGRGLQLLFYSVGIGQISSE	62
BV20S1A1N3T	AAGRGLQLLFYSIGIDQISSE	63
BV21S1	ILGQGPELLVQFQDESVVDDS	64
BV21S2A1N2T	NLGQGPELLIRYENEEAVDDS	65
BV21S3A1T	ILGQGPKLLIQFQNNGVVDDS	66
BV22S1A1T	ILGQKVEFLVSFYNNEISEKS	67
BV23S1A1T	GPGQDPQFFISFYEKMQSDKG	68
BV23S1A2T	GPGQDPQFLISFYEKMQSDKG	69
BV24S1A1T	KSSQAPKLLFHYYNKDFNNEA	70
BV24S1A2T	KSSQAPKLLFHYYDKDFNNEA	71
BV25S1A1T	VLKNEFKFLISFQNENVFDET	72
BV25S1A3T	VLKNEFKFLVSFQNENVFDET	73
AV1S1	YPGQHLQLLLKYFSGDPLVKG	77
AV1S2A1N1T	YPNQGLQLLLKYTSAATLVKG	78
AV1S2A4T	YPNQGLQLLLKYTTGATLVKG	79
AV1S2A5T	YPNQGLQLLLKYTSAATLVKG	80
AV1S3A1T	YPNQGLQLLLKYLSGSTLVES	81
AV1S3A2T	YPNQGLQLLLKYLSGSTLVKG	82
AV1S4A1N1T	SPGQGLQLLLKYFSGDTLVQG	83
AV1S5	HPNKGLQLLLKYTSAATLVKG	84
AV2S1A1	YSGKSPELIMFIYSNGDKEDG	85
AV2S1A2	YSGKSPELIMSIYSNGDKEDG	86
AV2S2A1T	YSRKGPELLMYTYSSGNKEDG	87
AV2S2A2T	YSRIGPELLMYTYSSGNKEDG	88
AV2S3A1T	DCRKEPKLLMSVYSSGNEDGR	89
AV3S1	NSGRGLVHLILIRSNEREKHS	90

AV4S1	LPSQGPEYVIHGLTSNVNNRM	91
AV4S2A1T	IHSQGPQYIIHGLKNNETNEM	92
AV4S2A3T	IHSQGPQNIIHGLKNNETNEM	93
AV5S1	DPGRGPVFLLLIRENEKEKRK	94
ADV6S1A1N1	SSGEMIFLIYQGSYDQQNATE	95
AV6S1A2N1	SSGEMIFLIYQGSYDEQNATE	96
AV7S1A1	HDGGAPTFLSYNALDGLEETG	97
AV7S1A2	HDGGAPTFLSYNGLDGLEETG	98
AV7S2	HAGEAPTFLSYNVLDGLEEKG	99
AV8S1A1	ELGKRPQLIIDIRSNVGEKKD	100
AV8S1A2	ELGKGPQLIIDIRSNVGEKKD	101
AV8S2A1N1T	ESGKGPQFIIDIRSNMDKRQG	102
AV9S1	YSRQRLQLLLRHISRESIKGF	103
AV10S1A1	EPGEGPVLLVTVVTGGEVKKL	104
AV11S1A1T	FPGCAPRLLVKGSKPSQQGRY	105
AV12S1	PPSGELVFLIRRNSFDEQNEI	106
AV13S1	NPWGQLINLFYIPSGTKQNGR	107
ADV14S1	PPSRQMILVIRQEAYKQQNAT	108
AV15S1	EPGAGLQLLTYIFSNMDMKQD	109
AV16S1A1T	YPNRGLQFLLKYITGDNLVKG	110
ADV17S1A1T	FPGKGPALLIAIRPDVSEKKE	111
AV18S1	ETAKTPEALFVMTLNGDEKKK	112
AV19S1	HPGGGIVSLFMLSSGKKKHGR	113
AV20S1	FPSQGPRFIIQGYKTKVTNEV	114
AV21S1A1N1	YPAEGPTFLISISSIKDKNED	115
AV22S1A1N1T	YPGEGLQLLLKATKADDKGSN	116
AV23S1	DPGKGLTSLLLIQSSQREQTS	117
AV24S1	DTGRGPVSLTIMTFSENTKSN	118
AV25S1	DPGEGPVLLIALYKAGELTSN	119
AV26S1	KYGEGLIFLMMLQKGGEEKSH	120
AV27S1	DPGKSLESLFVLLSNGAVKQE	121
AV28S1A1T	QEKKAPTFLFMLTSSGIEKKS	122
AV29S1A1T	KHGEAPVFLMILLKGGEQMRR	123
AV29S1A2T	KHGEAPVFLMILLKGGEQKGH	124

AV30S1A1T	DPGKGPEFLFTLYSAGEEKEK	125
AV31S1	YPSKPLQLLQRETMENSKNFG	126
AV32S1	RPGGHPVFLIQLVKSGEVKKQ	127

Exemplary combinations of TCR peptides that can be used in combination with low dose estrogen for preventing or reducing the severity of autoimmune diseases include, for multiple sclerosis, Vβ5.2, Vβ6.5 and/or Vβ13 (e.g. SEQ ID NOS:2, 3, 5 and/or 6); for rheumatoid arthritis, Vβ3, Vβ14 and/or Vβ17 (e.g. SEQ ID NOS:1, 7 and/or 8); and for psoriasis, Vβ3 and/or Vβ13 (e.g. SEQ ID NOS:1 and/or 6). Other appropriate combinations of TCR peptides can be determined by the skilled person according to the pathogenic TCRs expressed by the particular individual.

Alternatively, analogs of the T cell receptors and peptides can be used in the methods of the invention. As used herein, the term "T cell receptor analog" refers to a sequence with minor modifications, so long as the analog retains the ability to induce a substantially similar cell mediated or humoral immune response against the T cell receptor as the receptor or portion having the native sequence. A T cell receptor analog can thus have one, two or several amino acid deletions, additions or substitutions, with respect to the native sequence. For example, a T cell receptor analog can have a single amino acid substitution, or substitutions at 2, 3, 4 or more positions with respect to the sequences listed in Table 2. Such analogs can advantageously have improved stability, bioavailability, bioactivity or immunogenicity as compared to the native sequence. An exemplary analog of a Vβ sequence is the (Y49T) BV5S2-38-58 peptide having the amino acid sequence ALGQGPQFIFQTYEEEERQRG (SEQ ID

NO:3), which is a singly substituted analog of the naturally occurring BV5S2-38-58 peptide.

A TCR analog can have, for example, at least 70%, such as at least 80%, 90%, 95%, 98% or greater identity with the naturally occurring sequence over the entirety of the sequence. A TCR analog can be encoded by a nucleic acid sequence having at least 70%, such as at least 80%, 90%, 95%, 98% or greater identity with the naturally occurring sequence over the entirety of the sequence. Those skilled in the art can readily make and test peptide analogs, by either *in vitro* or *in vivo* assays, to determine whether they retain the immunological activity of the naturally occurring sequence. Additionally, computer programs that predict sequences containing B and T cell epitopes are known in the art, and can be used to guide the choice of amino acid substitutions, additions or deletions (see, for example, Savoie et al., Pac. Symp. Biocomput. 1999:182-189 (1999); Cochlovius et al., J. Immunol. 165:4731-4741 (2000)).

As shown in Example II, below, the combination of low dose estrogen and a Vβ8.2 peptide acted synergistically to result in complete protection against the autoimmune disease EAE. Thus, T cell receptor peptide therapies, as described above, can advantageously be combined with low dose estrogen therapy to reduce the severity of immune pathologies mediated by T cells expressing a limited repertoire of T cell receptors, including both autoimmune pathologies and T cell malignancies.

Alternatively, an expressible nucleic acid construct encoding an intact dimeric T cell receptor, a

full-length single T cell receptor chain, a variable region peptide or portion thereof, or hypervariable region peptide (e.g. the CDR2 region) or portion thereof, or analog of such sequences, can be administered to a mammal. The nucleic acid can be inserted into a plasmid vector, viral vector, or alternatively not be inserted into a vector. Those skilled in the art can determine the appropriate mammalian promoter and regulatory elements, route of administration and dose of nucleic acid required to induce an immune response against the pathogenic T cells. Preferred routes of administration of an expressible nucleic acid are intramuscular and intradermal. The use of expressible nucleic acid molecules encoding peptides to induce an immune response are described, for example, in U.S. Patent No. 5,580,859. The use of expressible nucleic acid molecules encoding T cell receptor peptides to elicit an immune response against pathogenic T cells is described, for example, in U.S. Patent Nos. 5,939,400, 6,113,903 and 6,207,645.

Similar immunotherapeutic methods as described above with respect to T cell receptors can be used to induce an immune response against an HLA molecule associated with an immune disease. For example, expression of HLA-DR1 and some subtypes of HLA-DR4 (eg. Dw4) are strongly associated with rheumatoid arthritis (RA); expression of HLA-B27 is strongly associated with ankylosing spondylitis and reactive arthritis; expression of HLA-DR15, DQ6 and Dw2 with multiple sclerosis (MS); HLA-DR3 and HLA-DR4 with diabetes; and HLA-DR2 and HLA-DR3 with lupus. Thus, HLA molecules associated with immune pathologies and characteristic portions thereof, and nucleic acid molecules encoding such polypeptides, can be used as immunotherapeutic agents in combination with estrogen therapy to reduce the severity of immune

pathologies. The association of HLA haplotypes with immune pathologies and methods of using HLA molecules as immunotherapeutic agents are described, for example, in U.S. Patent No. 6,045,796.

Immunomodulatory agents can advantageously be administered in combination with an adjuvant suitable for administration to the particular mammal. For humans, exemplary adjuvants include Incomplete Freund's Adjuvant, alum, and Detox™. Optionally, immunomodulatory agents can be conjugated to carrier molecules. Suitable adjuvants and carriers are well known in the art.

As used herein, an "immunoblocking agent" refers to any molecule that interferes with the interaction of the trimolecular complex between a T cell receptor, an HLA and an antigen. For example, an immunoblocking agent can be an antibody directed against and specific for a T cell receptor chain, such as specific for a rodent Vβ8.2, or human Vβ2, Vβ3, Vβ5.1, Vβ5.2, Vβ6.1, Vβ6.2, Vβ6.5, Vβ6.7, Vβ7, Vβ13, Vβ14 or Vβ17 chain. Methods of using antibodies as T cell receptor immunoblocking agents are described, for example, in Acha-Orbea et al., Cell 54:263-273 (1988) and U.S. Patent Nos. 5,223,426, 6,221,352 and 6,113,903.

Likewise, an immunoblocking agent can be an antibody directed against an antigen, such as the antigens associated with immune pathologies described above, or an antibody directed against an HLA antigen associated with an immune pathology, as described above.

As used herein, the term "antibody" refers to a polyclonal, monoclonal, chimeric or single chain antibody, or antigen-binding fragment therefrom (such as

as a Fab or Fab2 fragment), that binds an antigen with high affinity (Kd < about 10^5M) and high specificity. Methods of preparing antibodies specific for any given target molecule are well known in the art.

An immunoblocking agent can further by a complex of an antigenic peptide and an HLA molecule as described, for example, in U.S. Patent No. 5,194,425.

An immunoblocking agent can also be a non-antibody agent that specifically binds a desired target molecule on a pathogenic T cell (eg. T cell receptor, antigen or HLA). Libraries of naturally occurring and synthetic compounds, including inorganic compounds, peptides, lipids, saccharides, nucleic acids and small organic molecules, are commercially available, and can be screened in high-throughput assays to binding agents. Such agents can then be tested in *in vitro* or *in vivo* assays to determine their efficacy in blocking activation of pathogenic T cells.

For example, an immunoblocking agent can be an altered peptide ligand. As used herein, the term "altered peptide ligand" refers to an analog of an antigenic peptide (such as the autoantigenic peptides described above), in which the TCR contact residues have been altered, such that the peptide binds the HLA molecules with similar affinities as the wild-type peptide, but does not stimulate T cell proliferative responses. Methods of making and using altered peptide ligands of a variety of antigenic peptides are described, for example, in Evavold et al., Immunology Today 14:602-609 (1993), in Fairchild, Eur. J. Immunogenet. 24:155-167 (1997), and in U.S. Patent No. 6,197,926.

Advantageously, an antibody or other immunoblocking agent can further be attached to a toxic moiety, such as a chemotherapeutic agent or radioisotope to kill or inhibit proliferation of target cells. Such moieties and methods of attaching them to immunoblocking agents are known in the art.

An immunotherapeutic agent can alternatively be an agent that acts by a mechanism that is not specific for the trimolecular T cell receptor-antigen-HLA complex. Such agents include, for example, agents that modulate levels, production or function of cytokines, chemokines or their receptors. Those skilled in the art understand which sorts of agents will be effective in relation to different immune pathologies. For example, for treatment of Th1-mediated pathologies, useful agents include those that decrease Th1-type and/or increase Th2-type cytokine levels or activity.

Binding domains from the TNFα receptor (e.g. Enbrel™), or antibodies or other agents that bind to or block the function of TNFα (e.g. etanercept; infliximab), are exemplary immunotherapeutic agents that inhibit Th1 immune responses. Other agents include the naturally occurring IL-1 receptor antagonist (IL-1ra). Additionally, useful immunotherapeutic agents include general immunosuppressive agents such as corticosteroids, cyclosporine and FK506; anti-inflammatory cytokines such as IL-4, IL-10, TGF-β and interferons (e.g. interferon (IFN)beta-1a (Avonex™); IFNbeta-1b (Betaseron™); Rebif™); agents that non-specifically interfere with TCR/HLA/antigen interactions (e.g. the basic four-amino acid copolymer known as glatiramer acetate (Copaxone™)); antineoplastic agents (e.g. mitoxantrone (Novantrone™); purine analogs (e.g. 2-chlorodeoxyadenosine (cladribine);

2'-deoxycorfomycin (pentostatin)) as well as methotrexate, Cox-2 inhibitors (e.g. etoricoxib), phosphodiesterase inhibitors, leflunomide and the like, and various combinations of the above agents.

The invention also provides kits containing a low dose of estrogen and an immunotherapeutic agent, wherein administration of the low dose of estrogen and the immunotherapeutic agent reduce the severity of a Th1-mediated immune pathology in a mammal. Appropriate kit components and immunotherapeutic agents for treatment of various pathologies have been described above.

As used herein, the term "kit" refers to components intended for use together, which may be in the same or separate containers. An indication that components of a kit are for use together can be, for example, packaging of containers containing the components in a single package, or labeling either or both of the components as being for use in combination, or both. Such kits can further contain written instructions for use of the low dose estrogen formulation and immunotherapeutic agent in combination to reduce the severity of an immune pathology. Written instructions can, for example, set forth the clinical indication, as well as the amount, frequency, and method of administration of the kit components.

It will be appreciated that the estrogen formulation and agent formulation need not be the same. For example, the agent can be formulated for administration by injection, or other appropriate route, whereas the estrogen can be formulated for implantation, oral administration, inhalation or administration by another appropriate route. Other formulations for the

components of the kit can be determined by those skilled in the art, following the guidance provided above in relation to methods for reducing the severity of immune pathologies.

In addition to the active ingredients, the low dose estrogen and immunotherapeutic agent can be formulated with suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Further details on techniques for formulation and administration may be found in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, Pa.).

The kit components are provided in an effective amount to achieve the intended purpose. The determination of an effective dose is well within the capability of those skilled in the art. For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays, or in animal models, usually mice, rats, rabbits, dogs, or pigs. The animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

A therapeutically effective dose refers to that amount of active ingredient which ameliorates the symptoms or condition. Therapeutic efficacy and toxicity can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population)

and LD50 (the dose lethal to 50% of the population). The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50. Pharmaceutical compositions which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Factors which may be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions may be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation.

Normal dosage amounts of the immunotherapeutic agent may vary from 0.1 µg to 100 mg, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art.

The following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

Effect of low-dose estrogen therapy on an immune pathology

This example shows that administration of a low dose of either of two forms of estrogen, to either males or females, in two distinct animal models of multiple sclerosis, effectively reduced the incidence and severity of the disease. The reduction in clinical disease was accompanied by a significant decline in the number of inflammatory and demyelinating foci in the central nervous system. T lymphocytes from estrogen treated mice demonstrated a modest reduction in proliferation and a shift in cytokine production. Thus, low dose estrogen therapy is an effective method of reducing the severity of immune pathologies in mammals.

Materials and Methods

Animals. Age matched SJL/J and B10.PL mice were purchased from Jackson Laboratory (Bar Harbor, ME). Young adult (10 weeks old or less) mice were used for the experiments in this Example. The animals were housed in the Animal Resource Facility at the Portland Veterans Affairs Medical Center in accordance with institutional guidelines.

Antigens. Mouse proteolipid protein peptide 139-151 (HCLGKWLGHPDKF) (SEQ ID NO:74) and myelin basic protein peptide Ac1-11 (Ac-ASQKRPSQRSK) (SEQ ID NO:75) were synthesized using solid phase chemistry on a Synergy 432A

peptide synthesizer (Applied Biosystems, Foster City, CA), and purified prior to use.

Estrogen treatment and measurement of serum estrogen levels. Sixty-day release pellets of 17-β estradiol (E2), estriol (E3) and placebo pellets were implanted subcutaneously in the scapular region behind the neck using a 12 gauge trochar as described by the manufacturer (Innovative Research, Sarasota, FL). The mice were implanted one week prior to immunization with the appropriate myelin antigen. Representative animals were bled by cardiac puncture, and the blood was allowed to clot at 4°C overnight. The samples were centrifuged, the sera collected, and stored at -80°C until hormone analysis was performed. Serum levels of E2 and E3 were determined by radioimmunoassay (RIA) after Sephadex LH-20 column chromatography. All samples were analyzed in a single assay for each hormone.

Induction of EAE. SJL mice were inoculated subcutaneously in the flanks with 0.2 ml of an emulsion containing 150 µg of PLP 139-151 in saline and an equal volume of complete Freund's Adjuvant (CFA) containing 200 µg of Mycobacterium tuberculosis H37RA (Difco Laboratories, Detroit, MI). B10.PL mice were immunized with an emulsion containing 400 µg of MBP Ac1-11 and 200 µg of Mycobacteriurn tuberculosis. Disease induction in B10.PL mice required treatment with pertussis toxin on the day of immunization (75 ng/mouse) and 2 days later (200 ng/mouse). The mice were examined daily for clinical signs of disease and scored according to the following scale: 0, normal; 1, minimal or mild hind limb weakness; 2, moderate hind limb weakness or mild ataxia; 3, moderately severe hind limb weakness; 4, severe hind limb weakness or moderate ataxia; 5, paraplegia with no

more than moderate forelimb weakness; 6, paraplegia with severe forelimb weakness or severe ataxia.

Histopathology. The intact spinal column was removed from mice during the peak of clinical disease and fixed in 10% phosphate buffered formalin. The spinal cords were dissected after fixation, and embedded in paraffin prior to sectioning. The sections were stained with luxol fast blue-periodic acid schiff-hematoxylin and analyzed by light microscopy. Semi-quantitative analysis of inflammation and demyelination was determined by examining at least ten sections from each mouse.

Immunofluorescent staining for flow cytometry. Draining lymph node (DLN) cells were removed during the peak of clinical symptoms and analyzed for the expression of cell surface proteins by fluorescent staining *ex vivo*. The following fluorochrome conjugated antibodies obtained from Pharmingen Inc. (San Diego, CA) were used for the direct staining of DLN cells: anti-CD4, anti-CD25, anti-CD69, anti-CD95L, anti-CD44, anti-CD62L, anti-CD49d. Two-color immunofluorescent analysis was performed on a FACScan instrument (Becton Dickinson, Mountain View, CA) using Cellquest software. For each experiment the cells were stained with isotype control antibodies to establish background staining, and to set the quadrants prior to calculating the percent positive staining cells.

Proliferation Assays. DLN cells were recovered from immunized mice at peak of clinical EAE (days 12-16 post-immunization) as described in Bebo et al., J. Immunol. 162:35 (1998)). The *in vitro* proliferative response was determined using a standard microtiter assay (Bourdette et al., Cell Immunol. 112:351 (1988)). Briefly, DLN cells were cultured in 96 well, flat bottom

tissue culture plates at 4 x 10^5 cells per well in stimulation medium alone (control), or with test antigens (i.e. PLP 139-151) and incubated for 3 days at 37°C in 7% CO_2. Wells were pulsed for the final 18 hr with 0.5 mCi of [^{3}H] methylthymidine (Amersham, Arlington Heights, IL). The cells were harvested onto glass fiber filters and tritiated thymidine uptake measured by a liquid scintillation counter. Results were determined from the means of triplicate cultures. Stimulation indices were determined by calculating the ratio of antigen specific cpm to control cpm.

Cytokine detection by ELISA. DLN cells were cultured at 4 x 10^6/ml and stimulated with the appropriate antigen in 24 well culture plates. Cell culture supernatants were recovered between 48-72 hr and frozen at -70°C until needed for the cytokine assay. Measurement of cytokines was performed by ELISA using cytokine specific capture and detection antibodies (Pharmingen). Standard curves for each assay were generated using recombinant mouse cytokines (Pharmingen), and the concentration of cytokines in the cell supernatants was determined by interpolation from the appropriate standard curve. IFN-γ, TNF-α and IL-12 were chosen as representative Th1 cytokines, while IL-4 and IL-10 were measured as representative Th2 cytokines.

PLP 139-151 specific antibody ELISA. Nunc-Immuno 96 well ELISA plates (Nunc, Inc, Denmark) were coated with PLP 139-151 at 4 μg/ml in phosphate buffered saline (PBS) overnight at 4°C. The plates were washed and blocked prior to the addition of serum at the indicated dilution in triplicates. The samples were incubated overnight at 4°C and the plate was washed prior to the addition of an affinity purified, biotinylated goat anti-mouse Ig

(diluted 1:10,000) detecting antibody (Accurate Antibodies, Westbury, NY). The plates were incubated for one hour at room temperature before they were washed. A 1:400 dilution of avidin-peroxidase conjugate (Sigma, St. Louis, MO) was added to each well and the plates were incubated for an additional 45 mins. After the final wash, a peroxidase substrate (3,3',5,5-tetramethylbenzidine, Kirkgaard & Perry Laboratories, Gaithersburg, MD) was added to the wells and the reaction was stopped by the addition of 0.18 M sulfuric acid. The plates were read in a Vmax kinetic microplate reader (Molecular Devices, Inc., Sunnyvale, CA) at 450 nm. Wells coated with an irrelevant peptide (myelin oligodendrocyte glycoprotein 35-55) acted as a negative control.

Statistics. Cumulative disease index (CDI) was defined as the mean of the sum of the daily scores. Significant differences in disease incidence between placebo and estrogen treated mice were determined by chi square analysis and significant differences in disease onset, severity at peak of disease and CDI were determined using the two-tailed student t test.

<u>*Results*</u>

Low dose 17β-estradiol (E2) treatment reduces the incidence and severity of EAE in SJL mice. The protective effect of estrogen on severity of EAE, an animal model of multiple sclerosis was determined. Female SJL mice were implanted with 60-day release tablets (Innovative Research, Sarasota, FL) containing 17-estradiol (E2) one week prior to the active induction of EAE by immunization with proteolipid protein peptide 139-151 (PLP 139-151). The dose of E2 chosen for these

studies (Table 3) was intended to mimic the levels of E2 found during pregnancy, estrus or diestrus phases of the hormone cycle (Table 1). E2 levels were measured in representative animals and were determined to be equivalent to those reported by the manufacturer.

TABLE 3

Pellet (mg)	17β-estradiol (pg/ml)	Estriol (pg/ml)
15	9,000-10,000	10,000-15,000
5	3,000-4,000	5,000-6,000
2.5	1,500-2,000	2,000-3,000
1.5	800-1,000	800-1,000
0.36	150-200	150-200
0.1	25-50	40-50
0.025	5-10	10-20

As shown in Figure 1 and Table 4, pregnancy levels of E2 reduced the incidence and severity of clinical disease in a manner similar to that reported previously. Unexpectedly, low levels of E2 also profoundly reduced the clinical manifestations of disease. Pellets releasing as little as 25-50 pg of E2 per ml of serum lowered the incidence, delayed the onset, and significantly diminished the severity of paralysis when compared to placebo controls (Figure 1 and Table 4). In addition, pathological examination revealed a dramatic reduction in mononuclear cell infiltration and demyelination in the spinal cords of E2 protected mice when compared to placebo treated mice (Table 5).

TABLE 4

17β-estradiol (mg/pellet)	Incidence	Onset (days)	Relapse	Peak	CDI
Placebo	19/19 (100%)	12.3±0.4	5/10 (50%)	4.4±0.2	30.5±1.2
0.025 (diestrus)	15/18 (83%) p=0.201^{T}	15.8±3.5 p=0.09	5/9 (56%) p=0.843	3.6±1.3 p=0.27	19.2±10.0 p=0.07
0.1 (diestrus)	12/19 (63%) p=0.012	14.5±2.0 P=0.07	2/9 (22%) p=0.431	2.0±0.9 P=0.0002	8.9±3.4 P<0.0001
0.36 (estrus)	15/19 (79%) p=0.114	15.1±1.3 P=0.006	2/9 (22%) p=0.431	2.5±0.6 P<0.0001	9.8±1.9 P=0.0001
2.5 (pregnancy)	4/10 (40%) p<0.0001	13.8±0.5 P=0.02	0/4 (0%) p=0.252	2.0±2.1 P=0.06	7.5±7.9 P=0.003

TABLE 5

	Inflammatory foci/section	Demyelinated foci/section
Placebo	7.2±3.4	4.4±1.8
0.36 mg E2	1.3±0.8*	0.5±0.3
2.5 mg E2	0.6±0.8	0.5±0.4
2.5 mg E3	1.2±0.6	0.8±0.2
5.0 mg E3	0.9±0.5	0.3±0.2

Low dose estriol (E3) treatment reduces the incidence and severity of EAE in SJL mice. Estriol (E3) is a hormone produced by the placenta, and is at its highest levels during the third trimester. The effects of various doses of ES in the SJL EAE model was determined. As shown in Figure 2 and Table 6, high dose E3 therapy effectively

reduced the incidence and severity of EAE induced by active immunization of female SJL mice with PLP 139-151. Unexpectedly, low dose E3 treatment was also effective. Treatment of female mice with 1.5 mg E3 pellets resulted in serum hormone levels that were half to a third of that known to result from pregnancy (Tables 1 and 3). These mice had a lower incidence, delayed onset, and a significant reduction in the mean peak disease score and cumulative disease index (Figure 2 and Table 6). The diminution in clinical disease score was accompanied by a substantial reduction in inflammation and demyelination upon pathological examination (Table 5). The direct comparison of E2 and E3 in the same animal model also allowed determination of whether one form of estrogen was more or less potent than the other form. No statistically significant differences (as determined by the Fisher exact test) in the incidence or severity of EAE were found, indicating that E2 and E3 were equally protective.

TABLE 6

Estriol (mg/pellet)	Incidence	Onset (days)	Relapse	Peak	CDI
Placebo	10/10 (100%)	12.7±0.3	2/5 (40%)	4.2±0.2	30.2±1.5
1.5 (low)	6/10 (60%) p=0.094	16.4±0.7 P=0.02	0/3 (0%) p=0.673	2.0±0.0 P=0.004	9.3±2.6 P=0.008
5.0 (pregnancy)	2/5 (40%) p=0.040	33.5±5.0 P=0.03	0/2 (0%) p=0.895	1.4±2.2 P=0.31	6.5±9 p=0.07
15.0 (high)	5/10 (50%) p=0.039	23.8±9 p=0.22	0/1 (0%) NA	1.4±1.1 P=0.07	4.4±3.5 P=0.01

Low dose estrogen therapy reduces the incidence and severity of EAE in B10.PL mice. The effect of E2 and E3 on EAE was examined in B10.PL mice, which are genetically distinct from SJL mice and respond to a different dominant myelin antigen, myelin basic protein peptide Ac1-11 (MBP Ac1-11). The sensitivity of these mice to estrogen therapy was tested by treating the mice with estrogen containing pellets prior to immunization with MBP Ac1-11. Low level E2 treatment reduced the incidence, delayed the onset, and diminished the severity of EAE as reflected by significant differences in mean peak disease score and the cumulative disease index (Table 7).

TABLE 7

17β-estradiol (mg/pellet)	Incidence	Onset (days)	Peak	CDT
Placebo	26/30 (87%)	13.6±0.6	4.6±0.34	39.3±7.3
0.18 (diestrus)	3/6 (50%) p=0.125	25.7±1.2 P<0.0001	1.7±0.92 P<0.000	5.8±2.9 P<0.0001
0.36 (estrus)	15/26 (58%) p=0.030	26.5±1.3 P<0.0001	2.61±0.60 P<0.0001	13.5±6.2 P<0.0001
2.50 (pregnancy)	0/15 (0%) p<0.0001	0±0 NA	0±0 NA	0±0 NA
15.0 (high)	2/8 (25%) p=0.002	29±1.4 P<0.0001	2.0±1.0 P<0.0001	1.4±3.8 P<0.0001

When the cumulative disease indices and peak disease scores were compared (Fisher exact test), no significant differences in E2 sensitivity between SJL and B10.PL mice at low E2 levels were found . However B10.PL mice appeared to be more sensitive to high dose E2 treatment. Strain differences in peak disease score and

cumulative disease index were significant in mice receiving 2.5 mg E2 pellets ($p = 0.005$).

E3 also reduced the incidence and severity of disease in B10.PL mice (Table 8), but no differences in sensitivity to E3 were detected between these mice and SJL mice as determined by the Fisher exact test.

TABLE 8

Estriol (mg/pellet)	**Incidence**	**Onset (days)**	**Peak**	**CDI**
Placebo	3/4 (75%)	9.7±0.43	4.3±1.4	30.5±30.5
1.5 (low)	5/8 (38%) $p=0.551^{T}$	30.3±0.9 $P<0.0001$	1.38±0.8 $P<0.0001$	1.9±1.7 $P<0.0001$
5 (pregnancy)	3/8 (38%) $p=0.551$	31.3±0.7 $P<0.0001$	0.71±0.4 $P<0.0001$	1.0±0.5 $P<0.0001$

Male SJL mice are sensitive to estrogen. Estrogen receptors (ER) are expressed both by female and male immunocompetent cells. Because male cells are potentially sensitive to estrogens, estrogen therapy was performed on male SJL mice. Male mice were treated with E2 and E3 containing pellets as described above and one week later they were immunized with PLP 139-151. Treatment with either E2 or E3 delayed the onset and reduced the severity of clinical disease, even at doses equivalent to estrus levels (150-200 pg/ml) in females (Figure 3 and Table 9). No significant differences in estrogen sensitivity (as determined by the Fisher exact test) were detected between males and females.

TABLE 9

Treatment (mg/pellet)	Incidence	Onset (days)	Peak	CDI
Placebo	4/4 (100%)	11.3±0.5	4.4±0.8	25.4±6.5
0.36 E2	3/4 (75%) p=1.00[T]	15.3±3.2 P=0.052	2.1±2.4 P=0.119	6.4±5.1 P=0.004
2.5 E2	0/4 (0%) p=0.034	NA	NA	NA
2.5 E3	2/3 (67%) p=0.885	16.5±0.7 P<0.0001	0.67±0.6 P=<0.0001	3.2±2.5 P=0.003
5.0 E3	1/4 (25%) p=0.144	16.0±0 p<0.0001	0.50±1.0 P=<0.0001	2.6±5.3 P=0.002

Mechanisms governing estrogen mediated regulation of EAE. Alterations in the expression of adhesion or activation markers on T cells are often indicative of functional changes in the cell. Monoclonal antibodies specific for a number of these markers were used to assess whether estrogen therapy altered their expression. Draining lymph node (DLN) cells were recovered from mice during the peak of clinical EAE, incubated with the indicated fluorochrome conjugated monoclonal antibodies, and surface expression measured by fluorescent activated cell analyzer. As shown in Table 10, there were no apparent differences in the number of CD4+ T cells in the DLN from placebo and estrogen treated mice.

TABLE 10

	% of Total	% of CD4+ T cells					
	CD4	CD25	CD69	FASL	CD44	CD62L*	CD49d
Placebo	42*	5.0	7.2	2.8	38	7.2	36
0.36 E2	49	5.9	6.6	2.7	43	7.0	45
2.5 E2	49	6.3	6.5	4.2	41	6.7	43
2.5 E3	45	5.5	7.5	3.1	36	12	36
5.0 E3	47	6.4	8.3	2.7	38	8.8	37

*Representative of two different experiments.

Approximately 5% of the T cells in the DLN presented with an activated phenotype (CD25+, CD69+, FASL+), but no differences between placebo and estrogen treated mice was noted. In addition, no differences in adhesion molecule expression (CD44, CD62L, CD49d) were observed. These data indicate that estrogen therapy had no apparent effect on the phenotype of T lymphocytes in the lymph nodes draining the site of immunization.

Proliferation of draining lymph node (DLN) T cells from placebo and estrogen treated mice was measured to determine if estrogen therapy adhered the ability of these cells to respond to antigen. DLN T cells were removed from representative animals during the peak of clinical EAE, stimulated with antigen in vitro, and proliferation measured using a standard ^{3}H-thymidine incorporation assay. A modest decrease in proliferation to PLP 139-151 was consistently observed in DLN cells isolated from estrogen treated mice (Figure 4). However, in all cases the reduction in antigen specific proliferation failed to achieve statistical significance. No consistent differences in background, or mitogen-induced proliferation were observed. A similar modest

but insignificant reduction in antigen specific proliferation was also observed in the B10.PL model.

To determine if low dose estrogen therapy altered cytokine secretion patterns, DLN cells were prepared from individual mice at the peak of clinical EAE and cytokine levels were measured 48-72 hours after in vitro stimulation with PLP 139-151. IFN-γ, IL-12, and TNF-α were used as representative Th1 cytokines while IL-4, and IL-10 were used as representative Th2 cytokines. Even though the secretion of IFN-γ was consistently lower in E2 and E3 treated groups of mice (Figure 5A), the reduction in IFN- levels fell short of being statistically significant ($p > 0.10$). The decrease in IFN- secretion was accompanied by a modest increase in IL-10 (Figure 5B) and a small decrease in IL-12 (Figure 5E). Despite the lack of statistical significance, the trend towards higher Th1 and lower Th2 cytokines points towards a subtle shift in the Th1/Th2 balance. The shift can be seen more clearly when the cytokine response of each individual mouse is plotted as a ratio of IFN-γ to IL-10 (Figure 5C). There was a marked decrease in the frequency of high Th1 responder mice in the E2 and E3 treated groups when compared to placebo animals that approached significance ($p = 0.09$ for the 5.0 mg E3 treated mice). Additionally, no informative trends were detected in IL-4 secretion (Figure 5D), and TNF-α secretion was very often below the limits of detection for the assay (<31.25 pg/ml). Modest changes in cytokine responses induced by low dose estrogen therapy were also observed in the B10.PL model and were consistent with the data described for the SJL model.

The humoral immune response in low dose estrogen treated animals was compared to placebo controls. Serum was collected from individual mice at the peak of clinical disease and PLP 139-151 specific immunoglobulin levels were measured using a standard ELISA assay. No significant difference ($p \geq 0.180$) in PLP 139-151 specific antibody production was observed between placebo and estrogen treated groups (Figure 6). These data suggest that the modest shift towards Th2 cytokine production in estrogen treated mice was insufficient to enhance humoral immunity.

EXAMPLE II

Effect of the combination of low-dose estrogen and immunotherapy on an immune pathology

This example shows that the combination of vaccination with a BV8S2 (Vβ8.2) peptide and low-dose estrogen therapy resulted in full protection against disease in an animal model of multiple sclerosis, whereas only partial protection was observed with either therapy alone. Additionally, the combined effects of immunotherapy and low-dose estrogen therapy potentiated IL-10 production by regulatory T cells, and synergistically enhanced IL-10 and TGF production by antigen-specific T cells. Thus, low dose estrogen therapy is an effective method of enhancing the efficacy of immunotherapeutic agents in reducing the severity of immune pathologies in humans.

Materials and Methods

Animals. Tg mice bearing the functionally rearranged BV8S2 gene specific for MBP-NAc1-11 on the B10.PL background were provided by Dr. Joan Goverman (Seattle, WA). Male Tg mice were bred with B10.PL females, and the offspring tested for expression of the transgene by FACS analysis of blood cells stained for BV8S2 (Vβ8.2) as described in Goverman et al., Cell 72:551-560 (1993). Approximately half of each litter expressed the BV8S2 transgene, with approximately half of these transgenic littermates of each sex. For some experiments, mice expressing the BV8S2 transgene were compared to litter mates that did not express the transgene. The colony was housed and cared for at the Animal Resource Facility (Portland VAMC) according to institutional guidelines.

Antigens. N-acetylated MBP-1-11 peptide (Ac-ASQKRPSQRSK)(SEQ ID NO:75) was synthesized using solid phase techniques and was purified by high performance liquid chromatography (HPLC) at the Beckman Institute, Stanford University (Stanford, CA). Glutathione S-transferase (GST) and GST-BV8S2 proteins were expressed and purified as described in Vaniene et al., J. Neurosci. Res. 45:475-486 (1996). The GST-BV8S2 fusion protein contains the complete BV, BD, and BJ regions and the first 19 residues of the BC region from the TCR of an encephalitogenic rat T cell clone fused to the C-terminal end of GST. To control for the GST-BV8S2 protein, the GST protein was produced and purified using the same expression system. The GST protein was included as a control in all tissue culture experiments utilizing the GST-BV8S2 protein.

Induction of active EAE and protection with BV8S2 protein. EAE was induced in Tg male or female mice by injecting 400 g MBP-Ac1-11/CFA containing 200 g *Mycobacterium tuberculosis* s.c. over four sites on the flank. For TCR protection experiments, mice were injected with 12.5 µg recombinant rat BV8S2 protein/IFA (experimental) or saline/IFA or GST/IFA (sham controls) intraperitoneally (i.p.) on days -7 and +3 relative to injection of the MBP-NAc1-11, according to the protocol described in Kumar et al., J. Exp. Med. 178:909-916 (1993). In an alternative protocol, mice were given the initial two injections and then boosted weekly with 12.5 µg BV8S2 protein or saline given s.c. Groups of male and female mice that were treated with TCR protein (Figure 7) were litter mates.

Estrogen therapy. For estrogen hormone therapy or combined estrogen plus TCR therapy, 3mm pellets containing varying amounts of 17β-estradiol or estriol (Innovative Research of America, Sarasota, FL) were implanted subcutaneously on the animal's back seven days prior to induction of EAE. Control mice were sham operated but received no pellet. The estrogen pellets provide continuous controlled release of a constant level of hormone over a period of 60 days. The concentration of 17β-estradiol in pellets used in these experiments and the expected serum concentration of secreted hormone maintained in the mice are listed in Table 11, along with the established range of physiological serum hormone levels during the estrus cycle and pregnancy. Serum concentrations of estrogen monitored prior to and during the course of EAE in representative control and implanted mice consistently fell within the expected ranges.

TABLE 11

Pellet (mg)	17β-estradiol (pg/ml)	Physiological Equivalent
15	9,000-10,000	Pregnancy (5,000-10,000pg/ml)
5	3,000-4,000	
2.5	1,500-2,000	
1.5	800-1,000	
0.36	150-200	Estrus (100-200pg/ml)
0.10	25-50	Diestrus (20-30pg/ml)
0.025	5-10	

Disease assessment. Mice were assessed daily for clinical signs of EAE according to the 7-point scale described in Example I. The cumulative disease index (CDI) was determined for each mouse by summing the daily clinical scores, and the mean CDI ± SEM was calculated for the control and experimental groups. The mean clinical score (MCS) was calculated for each mouse by dividing the CDI by the duration (days) of disease, and the mean ± SEM calculated for the control and experimental groups.

Proliferation assay. Spleens (SPL) were removed surgically, and single cell suspensions were prepared. Proliferative responses of T cells were determined in 96-well microtiter plates by incubating 4 X 10^5 spleen cells plus antigen at an optimal concentration of 20μg/well. Cultures were incubated for 72 hr at 37°C and 7% CO_2, the last 18 hr in the presence of 0.5μCi ^{3}H-thymidine. Cells were harvested onto glass fiber filters, and thymidine uptake was determined by liquid scintillation. Mean cpm ± SEM were calculated from triplicate wells. The stimulation index (SI) was obtained by dividing cpm from antigen-stimulated wells by

cpm from wells with no antigen. SI in cultures stimulated with GST alone was subtracted from the SI induced with GST-BV8S2 protein.

Measurement of cytokine secretion. Spleen cells were suspended at 4 x 10^6 cells/ml in stimulation medium with and without specific antigens. Cell culture supernatants were recovered at 72 hr and frozen at -70°C until needed for the cytokine assay. Measurement of cytokines was performed by ELISA (Bebo et al., supra (1998)) using cytokine specific capture and detection antibodies (PharMingen, San Diego, DA). Capture antibodies for IFN-γ, IL-10, and TGF-β were diluted to 2 μg/ml in bicarbonate coating buffer (0.1M $NaHCO_3$, pH 8.2). Standard curves for each assay were generated using recombinant mouse cytokines (PharMingen), and the concentration of cytokines in the cell supernatants was determined by interpolation from the appropriate standard curve.

Assessment of antibody responses. Antibody reactivity to MBP-Ac1-11 peptide and GST-BV8S2 protein was determined by indirect ELISA as described in Hashim et al., J. Immunol. 144:4621-4627 (1990). Briefly, mouse antisera from treated and control Tg mice with EAE were incubated in antigen coated wells, and bound antibody was detected spectrophotometrically with peroxidase-labeled rabbit anti-mouse antibody and *o*-phenylene-diamine as a substrate. Differences between groups were determined using Student's t-test.

Ovariectomy. The ovaries were removed by making two bilateral incisions (5 mm) halfway between the base of the tail and the middle of the back, followed by small incisions (2.5 mm) through the peritoneal wall. The ovaries were pulled through the incisions by grasping the periovarian fat, the blood vessels ligated, and the ovaries removed. The incision was closed by surgical skin clips. The animals were allowed to recover for at least 1 week before initiation of experiments.

Androgen and estrogen detection. Mice were bled by cardiac puncture and the blood was allowed to clot at 4°C overnight. The samples were centrifuged, and the sera collected and stored at -80°C until hormone analysis was performed. Serum levels of estrogen were determined by radioimmunoassay (RIA) after Sephadex LH-20 column chromatography, as described in Roselli et al., Endocrine 64:139 (1996). All samples were analyzed in a single assay.

Results

Gender difference in treatment of EAE with BV8S2 protein. Responses to vaccination with BV8S2 protein were compared in male versus female Tg mouse littermates, using two different protocols. As is shown in Figure 7A and quantified in Table 12, males injected i.p. with BV8S2 protein/IFA on days -7 and +3 relative to EAE induction were significantly protected from EAE, with lower incidence and cumulative disease scores (CDI) than sham treated males. In contrast, females vaccinated using the same protocol were not protected from EAE (Figure 7C and Table 12). As illustrated in Figure 7B and 7E, the protective effect in males could be enhanced by boosting weekly with additional s.c. injections of BV8S2 protein.

These boosting injections had an early effect on littermate females as well, producing a significant delay in onset of clinical disease (Figure 7D and Table 12). However, this temporary suppression of clinical disease was lost abruptly (Figure 7D), and there was no significant amelioration of subsequent disease assessed during days 18-30 (Table 12).

TABLE 12

Group	Figure	Treatment Group	Incidence	Day of Onset	CDI	
Males	1A	Controls BV8S2	13/16 6/16*	15±1 14±1	49±10 17±7*	
Males	1B	Controls BV8S2,B	10/10 1/9***	13±1 15	63±9 7±7***	
Females	1C	Controls BV8S2	5/5 6/6	15±4 13±1	75±12 82±23	
Females	1D	Controls GST BV8S2,B	6/6 6/6 7/7	11±1 12±1 19±5**	Day 1-21 43±5 36±4 10±7**	Day 22-30 35±5 34±3 37±3

Effects of estrogen on EAE. The effects of sex hormones, including 17β-estradiol and estriol, were evaluated on the clinical course of EAE by hormone depletion or addition experiments. As shown in Figure 8A, female Tg mice unable to produce detectable levels of estrogen (<1pg/ml) or other sex hormones after ovariectomy developed significantly more severe EAE than sham ovariectomized females (CDI=81 versus 56, p<0.001). These data demonstrate that even basal levels of ovarian factors, possibly including estrogen, provide some regulation of EAE.

Treatment of sham or non-ovariectomized females with 17β-estradiol pellets produced a dose-dependent inhibition of EAE in both Tg females (Figure 8A) and B10.PL littermate females (Figure 8B). Notably, added estrogen had a less pronounced effect on the Tg versus non-Tg females. In B10.PL females, essentially complete inhibition of EAE was produced with 15mg pellets secreting pregnancy levels of 17β-estradiol (9,000-10,000pg/ml serum) over a 60 day period or with 2.5mg pellets (1,500-2,000pg/ml serum), and substantial inhibition was produced over a wide range of estrogen concentrations from estrus (0.36mg pellets secreting 150-200pg/ml serum) to diestrus levels (0.10mg pellets secreting 25-50pg/ml serum, Figure 8B). By comparison, in Tg females, pregnancy levels of hormone (15mg pellets) produced a marked but incomplete inhibition of EAE, with estrus levels (0.36mg pellets) producing only moderate inhibition (Figure 8A).

Estriol, which is normally elevated only during pregnancy, had an equivalent inhibitory effect on EAE in B10.PL females as 17β-estradiol (Figure 9B). The partial resistance to estrogen therapy in Tg females may be due to the higher native frequency of Ac1-11 specific T cells afforded by the transgene (Offner et al. supra (1998)) that likely accounts for an increased severity of EAE (CDI=56 in Tg vs 39 in non-Tg females, $p<0.001$). Consistent with this notion, estrogen treatment of ovariectomized female Tg mice reduced the severity of EAE to about the same level as sham ovariectomized Tg mice treated with estrogen (Figure 8A). However, the inability of estrogen to fully inhibit EAE in Tg females at very high levels approximating pregnancy suggests that a portion of the encephalitogenic cascade is estrogen insensitive.

Combined TCR and estrogen therapy. Because TCR and estrogen therapy were both partially effective for preventing EAE in Tg females, the effects of single versus combined therapy were directly compared. Consistent with the results described above, Tg females vaccinated with BV8S2 protein (with weekly boosting) had delayed onset but eventually developed severe EAE, whereas mice treated with estrus levels of E2 (0.36mg pellets secreting 150-200pg/ml serum over 60 days) had normal onset but generally less severe disease (Figure 9). However, combined treatment with both TCR protein and estrus levels of E2 produced almost complete protection against EAE (Figure 9), with only 3 of 16 mice developing very mild disease (Table 13). A similar degree of protection was provided in ovariectomized Tg females treated with BV8S2 protein and estrogen (Table 13), demonstrating that the enhanced therapeutic effect was dependent on E2 rather than a combination of estrogen and other gonadally produced sex hormones.

TABLE 13

Total	Incidence	Onset	Peak	Mortality	Average CDI (10-30)
Control	60/64	11.8±1.1	4.7±0.1	12/49	56.4±7.0
BV8S2	45/49	17.4±2.2^{A}	3.9±1.1^{A}	1/49	32.0±13.2^{A}
17β-estradiol	25/33	15.1±2.1A	3.6±0.7^{A}	1/33	37.3±6.2^{A}
BV8S2-17β-estradiol	3/16	18.8±8.0^{A}	0.5±0.4^{A}	1/16	3.1±4.0^{A}
Ovx	27/27	10.6±0.9	5.3±0.7	7/27	81.2±17.3^{A,C}
BV8S2	23/26	18.0±1.9	3.9±1.3	4/26	40.1±20.9^{B}
17β-estradiol	11/15	13.6±1.5	3.5±0.8	4/15	40.0±22.1^{B}
BV8S2+17β-estradiol	3/16	20.8±6.0	0.9±0.9	0/16	7.4±6.7^{A,B}

Data are combined from a total of 10 separate experiments. ^{A}Significant difference between control and experimental ($P<0.0001$). ^{B}Significant difference between Ovx control and Ovx experimental ($P<0.0001$). ^{C}Significant diifference between Ovx and non-Ovx control ($P<0.001$). Ovx, ovarectomized.

Estrogen skews response to Ac1-11 and potentiates response to BV8S2 protein. To investigate the mechanism(s) involved with individual and combined therapies, proliferation and cytokine responses of immune T cells from naive and treated mice were evaluated. As shown in Figure 10A, BV8S2 naive Tg males and females had equivalent proliferation responses to Ac1-11 peptide, PPD, and ConA, but naive Tg females had a strikingly reduced reactivity to the BV8S2 protein. This finding suggests that Tg females have a diminished native capacity to regulate an encephalitogenic response.

During development of EAE, splenic T cell responses to Ac1-11 peptide were characterized by moderate proliferation and production of TGF-β, and essentially absent secretion of IL-10 (Figure 10B). Secretion of IFN-γ in response to Ac1-11 peptide was modest, reflecting preferential migration of inflammatory T cells to draining lymph nodes and the CNS as observed previously(Offner et al. supra (1998)). Treatment with either BV8S2 protein or estrogen alone reduced proliferation and marginally affected cytokine responses to Ac1-11. However, combined treatment with both BV8S2 protein and estrogen markedly reduced proliferation and dramatically enhanced production of IL-10 and TGF-β, but not IFN-γ in response to Ac1-11 peptide (Figure 10B).

In contrast, splenic proliferation and IL-10 responses to BV8S2 protein were enhanced by both treatments individually, and further potentiated with combination therapy, with no significant effects of treatment on IFN- and TGF- secretion (Figure 10C).

Additionally, combination therapy reduced circulating levels of Ac1-11 specific IgG2a antibody associated with Th1 response, with no effect on IgG1 response (Figure 10D).

EXAMPLE III

17β-estradiol inhibits chemokines and receptors in EAE

This example shows that the protective effect of low dose estrogen on animals with a Th1 immune pathology is mediated, in part, by inhibition of mRNA expression of chemokines, chemokine receptors, and inflammatory cytokines by recruited inflammatory T cells.

Materials and Methods

Mice. Transgenic mice were obtained, bred and housed essentially as described in Example II. Mice were used at 8-12 weeks of age.

Induction of active EAE. EAE was induced, and disease assessed, essentially as described in Example II. Disease onset was defined as the first day of clinical signs, peak - acute phase of EAE - as maximum severity of clinical signs (day 16-17 after immunization with encephalitogenic peptide) and recovery as day 28 post-immunization, when clinical severity of EAE was diminished. The cumulative disease index (CDI) was determined for each mouse by summing the daily clinical scores. Mice selected from control, ovariectomized, and 17β-estradiol treated groups were sacrificed and spinal cords were isolated by insufflation and frozen at -70C or mononuclear cells were isolated over a Percoll step gradient and counted as described in Offner et al., J.

Immunol. 161:2178-2186 (1998). Lymph nodes (LN) or spleens (SPL) were removed surgically and passed through a wire mesh screen to obtain a single-cell suspension. Frozen spinal cords were subsequently thawed and evaluated for expression of chemokines, chemokine receptors and cytokines by the RNase protection assay.

17β-estradiol treatment. For 17β-estradiol hormone therapy, 3-mm pellets containing 0.36 mg of 17β-estradiol (Innovative Research of America, Sarasota, Florida), expected to provide physiological equivalence of the estrus cycle (150-200 pg/ml in serum) over 60 days, were implanted s.c. on the animal's back 7 days before induction of EAE. Control mice were sham operated and implanted with a pellet containing saline (which did not affect the course of EAE) or no pellet. The 17β-estradiol pellets provide continuous controlled release of a constant level of hormone over a period of 60 days. Serum concentrations of 17β -estradiol were evaluated in representative mice from each group. Sham treated mice had variable levels of 17β-estradiol that reflected both estrus and diestrus mice (ranging from about 20 to 200 pg/ml, on average about 50 pg/ml). In contrast, OVX mice did not have any detectable estradiol (<1 pg/ml). E2-treated intact mice were also variable (about 200-400 pg/ml), reflecting the combination of native estrus/diestrus levels plus the exogenous estradiol from the pellet, whereas the E2-treated OVX mice were somewhat lower (about 150-200 pg/ml).

RNase protection assay. Total RNA was extracted from frozen spinal cords or lymph node cells using the STAT-60 reagent (Tel-Test, Inc., Friendswood, TX). Chemokine expression was determined by using the RiboQuant RPA kit (PharMingen) according to the manufacturer's

instructions. A multiprobe set detected the following chemokine transcripts: C-X-C chemokines: MIP-2 and IP-10; C-C chemokines: RANTES, MIP-1α, MCP-1, and TCA-3; and C chemokine: Ltn. The chemokine receptor set detected the following transcripts: CCR1, CCR1b, CCR2, CCR3, CCR4, CCR5. Using RPA multiprobe it was possible to detect the following cytokines: IL-4, IL-10, TNF-α, LTβ, IFN-γ. The sample loading was normalized by the housekeeping gene, L32, included in each template set. RPA analysis was performed on 10 μg total RNA hybridized with probes labeled with [^{32}P]UTP. After digestion of ssRNA, the RNA pellet was solubilized and resolved on a 5% sequencing gel. Controls included the probe set hybridized to transfer RNA only, appropriate control RNA which serves as integrity control for the RNA sample, and yeast tRNA as a background control. For quantification, gels were exposed by phosphorimaging (Bio-Rad Laboratories, Hercules, CA) and radioactivity in individual bands (after background subtraction) in comparison with L32 was assessed with Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Proliferation assay. Proliferation responses of splenic T cells were determined essentially as described in Example II, except that antigen was used at an optimal concentration of 50 μg/ml.

Flow cytometry. Lymph node cells from sham and 17β-estradiol treated animals with EAE were washed with PBS/2%FCS/0.2%NaN_3 and first incubated on ice for 30 min with Fcγ III/II receptor blocking monoclonal antibody (PharMingen), then stained with PE-conjugated anti-CD3 (PharMingen). After 20 min of incubation with anti-CD3, the cells were fixed and permablized using Cytofix/Cytoperm (PharMingen). Subsequently, cells were

incubated with anti-CCR1, anti-CCR2, anti-CCR3, anti-CCR4 and anti-CCR5 goat anti-mouse polyclonal antibodies (Santa Cruz Biotechnology Inc.; USA) for 30 min on ice. After washing, cells were stained with FITC-conjugated anti-goat monoclonal antibodies (Sigma) for an additional 30 min on ice. Cells were analyzed using a FACS-Scan (Becton Dickinson). Propidium iodide and forward/side scatter gating were used to exclude dead cells.

Measurement of cytokine secretion. Lymph nodes of naïve BV8S2 TCR transgenic females were suspended at 4×10^6 cells/ml in stimulation medium with antigen and with or without 2000pg/ml of 17β-estradiol. Cell culture supernatants were recovered at 72 hours and frozen at -70C until used. Measurement of cytokines was performed by ELISA essentially as described in Example II.

Ovariectomy. Ovariectomy was performed essentially as described in Example II.

Statistics. Non-parametric clinical EAE data (peak disease scores and cumulative disease index) were evaluated between groups using the Mann-Whitney test; the day of onset among the various groups was evaluated using the t-test or ANOVA; the incidence and mortality rates were compared using the 2 test (Fisher's exact test in some instances). Comparison of RPA values, cytokine values, and CPMs were evaluated by the t-test or ANOVA. The accepted level of significance was $p<0.05$.

<u>*Results*</u>

Effects of 17β-estradiol on EAE. 17β-estradiol (E2) or estriol released from implanted pellets was shown in Example I to partially inhibit EAE in a dose dependent

manner in BV8S2 transgenic mice. Clinical EAE data for groups of these mice used in the current study are graphed in Figure 11 and summarized in Table 14. Mice implanted with 0.36 mg pellets of 17β-estradiol, which in combination with native hormone (20-200 pg/ml in sham mice) provided 150-400 pg/ml E2 in serum (estrus levels), developed significantly later onset and less severe EAE (lower peak score and CDI) than sham operated control mice (Figure 11A and Table 14). In addition to reducing disease severity, 17β-estradiol treatment inhibited proliferation of MBP-Ac1-11 specific T cells by an average of about 47%, and prevented infiltration of mononuclear cells into spinal cords by approximately 60% (Table 15).

In contrast, ovariectomized mice, in which endogenous 17β-estradiol was not detectable (<1 pg/ml), developed significantly earlier onset and more severe signs of EAE than sham operated mice (Figure 11B and Table 14). 17β-estradiol treatment of ovariectomized mice with implanted 0.36 mg pellets provided about 100-200 pg/ml E2 in serum, and inhibited EAE to approximately the same degree as E2-treated non-ovariectomized mice (Figure 11C and Table 14).

TABLE 14

Group of mice	Incidence	Onset	Peak	CDI
Sham	19/19	11.4±0.5	4.3±0.3	64.9±4.7
17β-estradiol treated	15/18	17.4±1.8*	2.5±0.5**	40.4±7.1**
Sham	12/12	11.3±0.7	3.8±0.5	59.4±4.9
OVX	12/12	9.8±0.6*	4.8±0.4*	85.3±7.3**
OVX	12/12	9.8±0.6	4.8±0.4	85.3±7.3
OVX+17β-estradiol[a]	8/11	16.5±2.3**	2.6±0.6**	40.0±8.5**

* $p<0.05$

** $p<0.01$

[a] EAE severity also significantly less than in Sham group

TABLE 15

Group of mice	Mean clinical score*	Number of cells/cord
Sham	2.4	27000
17β-estradiol treated	1.3	10000

*mean clinical score at time of cord removal

17β-estradiol treatment results in downregulation of chemokine mRNA expression in spinal cords. To optimize detection of clinically-related changes, chemokine expression was compared in 17β-estradiol protected versus sham pellet implanted mice during the peak acute phase of disease (16-17 days after immunization with MBP-Ac1-11 peptide/CFA) and during the chronic phase (28 days after immunization). At the earlier time point, spinal cord (SC) tissue was sampled from sham-treated mice exhibiting paralytic EAE (scores of 4-5) and compared to SC tissue from 17β-estradiol treated mice which had not yet

developed any clinical signs (EAE scores of 0). A quantitative method - the RNase protection assay - was employed to examine RNA synthesis for the following chemokines: RANTES, MIP-1α, MCP-1, and TCA-3 (C-C - subfamily); and MIP-2 and IP-10 (C-X-C - subfamily). The tissue sample for RPA was prepared by homogenization of whole spinal cord and total cellular RNA was extracted.

As quantified in Figure 12, transcripts for RANTES, MIP-1α, MIP-2, IP-10 and MCP-1, but not TCA-3 were detected in SC from Tg females at the peak and chronic phases of disease. Paraplegic mice (sham group) had abundant RNA expression of RANTES, IP-10, and MCP-1, with lesser mRNA levels of MIP-1α and MIP-2 (Figure 12). 17β-estradiol protected mice had profoundly lower levels of mRNA expression of all detectable chemokines. At the peak of disease, the difference between groups reached $p<0.001$ (Figure 12). Differences in chemokine expression between sham and E2-treated groups were still present but less pronounced during the chronic phase of EAE, reflecting the clinical status of the donors (sham, EAE scores of 3; E2, EAE scores of 0-1).

Ovariectomy increases mRNA expression of MIP-1α and MIP-2. Ovariectomy resulted in loss of detectable 17β-estradiol, as well as other ovarian hormones, and significantly enhanced the clinical severity of EAE (Figure 11A and Table 14), implicating basal levels of these factors in natural regulation. To evaluate effects of hormone depletion on chemokine levels during EAE, an RPA analysis was carried out of SC from ovariectomized female mice on day 16 after induction of EAE. SC were sampled from sham-treated mice with EAE scores of 4-5, and from ovariectomized mice with EAE scores of 5-6). Surprisingly, ovariectomized mice that displayed the most

severe signs of EAE had lower mRNA levels than sham treated mice of the normally predominant chemokines RANTES, IP-10 and MCP-1, but significantly enhanced expression of MIP-1α and MIP-2 (Figure 12). 17β-estradiol treatment of ovariectomized females (that inhibited EAE to a comparable degree as E2-treatment of intact mice), strongly inhibited expression of all detectable chemokines (Figure 12), again reflective of the sampling of SC from mice that had not yet developed overt clinical disease (EAE scores of 0). These results demonstrate the capacity of supplemental 17β-estradiol to profoundly inhibit chemokine expression, and implicate ovarian factors, including 17β-estradiol, as natural regulators of MIP-1α and MIP-2.

MIP-1α and MIP-2 are produced by infiltrating mononuclear cells in CNS. To discern which chemokines were produced by infiltrating cells within the CNS, expression of chemokines in whole CNS tissue versus isolated CNS mononuclear cells in Sham treated mice at the peak of EAE were compared. As quantified in Figure 13, message for MIP-1α and MIP-2, but not RANTES, IP-10 and MCP-1, was enriched in the CNS mononuclear cell fraction, whereas message for RANTES was reduced in the mononuclear cell fraction.

17β-estradiol therapy reduces chemokine receptor mRNA expression in CNS. In addition to chemokines, expression of chemokine receptors in spinal cords of 17β-estradiol treated and control BV8S2 transgenic mice during EAE was assessed. As quantified in Figure 14, message for CCR1, CCR2 and CCR5 was clearly enhanced in SC samples during both peak and chronic phases of EAE, whereas message for CCR1b, CCR3 and CCR4 was not detectable at either time point. 17β-estradiol treatment that prevented EAE

strongly inhibited the elevated mRNA levels for CCR1, CCR2 and CCR5 observed in sham treated mice with EAE ($p<0.001$). Ovariectomized mice had reduced levels of message for CCR1 and CCR2, with a lesser effect on CCR5 compared to sham-implanted control mice, and supplemental 17β-estradiol treatment again inhibited expression of all of these chemokine receptors (Figure 14).

Down-regulation of CCR1 and CCR5 in lymphocytes isolated from peripheral lymph nodes of females treated with 17β-estradiol. The reduction of chemokine receptors in SC of 17β-estradiol treated mice raised the possibility that there might be a systemic effect of 17β-estradiol therapy on chemokine receptor expression by lymphocytes. Thus, chemokine receptors on lymph node cells from 17β-estradiol versus sham treated mice with EAE were quantified using specific antibody staining and FACS analysis. Lymph node CD3+ T cells from 17β-estradiol treated mice had reduced mean channel fluorescence and a significantly lower percentage of positive cells when stained with antibodies to CCR1 (77+2 vs 91+6%, $p=0.02$) and CCR5 (27+2 vs 35+4, $p=0.03$) compared to T cells from sham treated mice. No difference was observed in CCR2 staining, and CCR3 and CCR4 were not detectable.

17β-estradiol down-regulates Th1 cytokines but does not cause Th2 cytokine switch. Two possible effects of 17β-estradiol therapy are 1) direct inhibition of inflammatory cytokines or 2) enhancement of Th2 cells and cytokines (Th2 switch) that could locally inhibit Th1 cells. RPA analysis of cytokine message revealed predominant expression of LT-β, TNF-α, and IFN-γ in the SC of mice at the peak of EAE that was significantly inhibited in 17β-estradiol treated mice (LT-β, $p<0.02$; TNF-α, $p<0.0001$; IFN-γ, $p<0.01$, Figure 15). In contrast,

message for IL-4 and IL-10 was not detectable in SC of mice with EAE, nor were these cytokines induced by 17β-estradiol treatment, indicating no Th2 switch in CNS. No changes in cytokine expression were noted in lymph node cells from E2-treated versus sham control groups.

17β-estradiol exerts little effect on T cells cultured in vitro. To evaluate 17β-estradiol effects on antigen specific T cells, splenocytes from naïve BV8S2 single transgenic female mice were stimulated with MBP-Ac1-11 peptide or BV8S2 protein in the presence or absence of a range of 17β-estradiol concentrations. As is shown in Figure 16A, 15-2,000 pg/ml 17β-estradiol had no significant effect on native T cell proliferation response to the encephalitogenic MBP-Ac1-11 peptide, and a modest enhancing effect on the native response to the BV8S2 protein. A relatively high dose (2,000 pg/ml) of 17β-estradiol mediated a 50% reduction of secreted IFN-γ protein, with no effects on IL-12 or IL-10 secretion, by MBP-Ac1-11 stimulated LN cells from naïve BV8S2 transgenic mice ($p<0.005$, Figure 16B). However, lower doses of 17β-estradiol comparable to serum levels in the mice studied above (150-400 pg/ml) had no inhibitory effects on IFN-γ or other cytokine secretion *in vitro*.

EXAMPLE IV

Low dose estrogen down-regulates TNFα production

This example shows that the protective effect of low dose estrogen on animals with a Th1 immune pathology is mediated, in part, by down-regulation of TNF-α secretion at the site of the pathology.

Materials and Methods

Mice. Female C57BL/6, IL-4 KO (B6.129P2-Il4^{tm1Cgn}), IL-10 KO (C57BL/6-Il10^{tm1Cgn}), and IFN-γ KO (B6.129S7-Ifng^{tm1Ts}) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in the Animal Resource Facility at the Portland Veterans Affairs Medical Center in accordance with institutional guidelines.

Antigens. Mouse myelin oligodendrocyte glycoprotein (MOG) 35-55 (MEVGWYRSPFSRVVHLYRNGK (SEQ ID NO:76)) was synthesized using solid phase techniques and purified by high performance liquid chromatography (HPLC) at the Beckman Institute, Stanford University (Palo Alto, CA).

Estrogen treatment. Sixty-day release pellets containing 2.5 mg of 17β-estradiol (E2) or vehicle were implanted subcutaneously (s.c) in the scapular region behind the neck using a 12-gauge trochar as described by the manufacturer (Innovative Research of America, Sarasota, FL). The mice were implanted one week prior to immunization with MOG 35-55. The concentration of E2 expected in the serum is between 1,500-2,000 pg/ml, which is approximately 5 times less than the levels found during pregnancy. E2 levels measured previously as described in Example I were found to be equivalent to those reported by the manufacturer.

Induction of EAE. C57BL/6 and cytokine deficient mice were inoculated s.c in the flanks with 0.2 ml of an emulsion containing 200 µg of MOG 35-55 in saline and an equal volume of complete Freund's Adjuvant (CFA) containing 400 µg of Mycobacterium tuberculosis H37RA (Difco Laboratories, Detroit, MI). Disease induction required i.v. administration of pertussis toxin on the

day of immunization (25 ng/mouse) and 2 days later (67 ng/mouse). The mice were assessed daily for clinical signs of EAE according to the 7 point scale described in Example I.

Histopathology. Histopathologic assessment of spinal column section was performed essentially as described in Example I, except that sections were stained with either luxol fast blue-periodic acid schiff-hematoxylin or silver nitrate prior to analysis.

RNAse protection assay. Chemokine, chemokine receptor and cytokine mRNAs were detected by RPA essentially as described in Example III, except that analysis was performed on 20 μg total RNA.

Proliferation assay. Proliferation assays were performed on draining lymph node (DLN) and spleen (SP) cells essentially as described in Examples I and II, using MOG 35-55 as the test antigen.

Intracellular staining for cytokines. Single cell suspensions from spleen were prepared from immunized mice and cultured at 10 x 10^6 cells/ml in stimulation media containing 50 μg/ml of MOG 35-55. The cells were stimulated for 24 hrs, the last 5 hrs in the presence of Brefeldin A. The cells were then stained with anti-Vβ8.1/8.2 TCR FITC for 30 min at 4°C prior to fixation and permeabilization with cytofix/cytoperm solution (Pharmingen, Inc.). The cells were then stained with anti-cytokine antibodies labeled with phycoerythrin (anti-mouse IFN-γ, TNF-α, IL-4, IL-10, and IL-12 from Pharmingen Inc.) for 30 min at 4°C. The cells were washed twice in perm/wash buffer (Pharmingen, Inc.) and once in FACS staining buffer (PBS, 1%BSA, 0.05% NaN_3) prior to

two-color FACS analysis on a FACScan instrument (Beckton-Dickenson, Inc., Sunnyvale, CA) using Cell Quest software (Beckton-Dickenson, Inc.). For each experiment the cells were stained with isotype control antibodies to establish background staining and to set the quadrants prior to calculating the percent positive cells.

CNS mononuclear cells were isolated from perfused brain and spinal cord by percol gradient centrifugation as described in Bebo et al., J. Neurosci. Res. 52:420-429 (1998). The cells were stimulated with MOG-35-55 peptide for 24 hrs, the last 5 hrs in the presence of Brefeldin A. The cells were then stained with anti-CD4 cychrome labeled antibodies prior to fixation and permeabilization. The cells were subsequently stained with Vβ8.1/8.2 TCR-FITC and the indicated cytokine specific antibody coupled to PE and analyzed by three-color flow cytometry. For each experiment the cells were stained with isotype control antibodies to establish background staining and to set the quadrants prior to calculating the percent positive cells.

Statistical analysis. Significant differences in incidence and mortality between untreated and E2 treated mice were assessed by Chi-square analysis. Difference in onset was determined using the two-tailed Student t test. Differences in peak score and cumulative disease index (CDI) were assessed by the Mann-Whitney test. Statistical significance of the frequency of cytokine secreting cells was analyzed using Student's t test for comparisons of two means. Differences in the expression of chemokine and cytokine mRNA were also determined using the Student t test. Values of $p < 0.05$ were considered significant.

Results

Estrogen treatment reduces the severity of EAE in C57BL/6 and cytokine deficient mice. The role of regulatory cytokines in estrogen-induced protection from EAE was examined using cytokine deficient mice. As described in Examples I-III, immunization with MOG 35-55 resulted in the induction of severe EAE in wild type (WT) C57BL/6 mice. No differences in disease severity were found in similarly immunized cytokine deficient mice (Figure 17 and Table 16). C57BL/6 mice implanted with 17β-estradiol (E2)-containing pellets had a lower incidence of EAE, and developed disease much later than untreated mice. However, EAE that eventually developed in some E2-treated mice was essentially equivalent in severity to untreated animals, possibly due to early depletion of the E2 pellets. Nevertheless, treatment with E2 exerted a profound reduction in both the incidence and cumulative disease index (CDI) of EAE, and significantly delayed onset of symptoms in those mice that eventually developed disease. Estrogen treatment had similar effects on mice deficient in IL-4, IL-10 and IFN-γ (Figure 17 and Table 16). No statistically significant differences in the ability of E2 to protect cytokine deficient mice were found (as determined by the Fisher exact test).

TABLE 16

	E2	Incidence	Onset	Mortality	Peak Score	CDI
B6	-	29/31	10.9±1.9	3/31	5.1±0.9	64.7±27.0
	+	19/31	20.8±3.7	0/31	4.2±1.2	15.1±17.0
		p=0.005*	P<0.0001	p=0.238	P=0.222	P<0.0001
IL-4	-	11/11	11.5±2.3	3/11	5.5±0.4	76.7±20.1
KO	+	8/11	20.6±4.9	0/11	4.4±1.0	18.4±22.2
		p=0.214	P<0.0001	p=0.214	P=0.561	P<0.0001
IL-10	-	15/16	12.3±1.3	2/16	4.9±1.1	53.0±27.2
KO	+	6/17	22.0±4.3	0/17	4.2±0.8	6.5±10.9
		p=0.001	P<0.0001	p=0.227	P=0.977	P<0.0001
IFNγ	-	11/11	13.6±2.6	1/11	4.9±1.1	58.6±22.4
KO	+	8/10	21.8±2.7	1/10	4.5±1.2	19.3±15.9
		p=0.214	P<0.0001	p=1.000	P=0.999	P<0.018

MOG 35-55 immunized C57BL/6 and cytokine deficient mice had numerous inflammatory and demyelinating lesions in the spinal cord at the peak of EAE, and no significant differences in the number and size of the lesions were observed. Healthy C57BL/6 and cytokine deficient mice that were treated with E2 before immunization did not have any detectable lesions in the spinal cord (Table 17). Thus, it is apparent from these data that E2 can suppress the development of both the clinical and histopathological manifestations of EAE in the absence of IL-4, IL-10, or IFN-γ.

TABLE 17

	Treatment with E2	Inflammatory Foci/Section*
C57BL/6	+ -	0.0±0.0 5.7±1.7
IFN-γKO	+ -	0.0±0.0 5.7±4.1
IL-4KO	+ -	0.0±0.0 7.0±1.5
IL-10KO	+ -	0.0±0.0 8.0±1.9

Inflammatory foci were enumerated from between 7-10 sections per spinal cord, at least two spinal cords were examined per group.

Estrogen treatment reduces chemokine and chemokine receptor mRNA expression in the CNS. The egress of inflammatory cells into the CNS is a critical first step in the development of EAE. Chemokines are low molecular weight chemotactic molecules that are thought to play an important role in the migration and retention of immunocompetent cells in the CNS . The influence of E2 treatment on chemokine and chemokine receptor mRNA in the spinal cords of WT and cytokine deficient mice was measured using the RNAse protection assay (RPA). Total RNA was purified from spinal cords collected from mice at the peak of EAE (day 12-16 post-immunization) and chemokine/chemokine receptor specific mRNA was detected using radiolabeled riboprobes. mRNAs coding for many of the chemokine and receptor family members were detectable in the spinal cords of WT C57BL/6 mice with EAE (Figure 18). RANTES and IP-10 were expressed at the highest levels, followed by MIP-1α, MIP-2 and MCP-1. The levels of TCA-3 mRNA were below the limits of detection for this assay. CCR5 was the most abundant chemokine receptor,

followed by CCR1 and CCR2 (Figure 18), whereas CCR1b, CCR3, and CCR4 were below the level of detection.

The expression of chemokine and chemokine receptor mRNA in cytokine deficient mice with EAE was often markedly different from that in WT mice (Figure 18). IL-4 deficient mice had reduced expression of RANTES and MIP-1α, but increased expression of MCP-1, whereas IL-10 and IFN-γ deficient mice had reduced expression of all chemokines tested except MCP-1. Of interest, TCA-3 mRNA was only detectable in INF-γ deficient mice. The expression of CCR1, CCR2, and CCR5 was nearly absent in IL-10 deficient mice, but was only moderately altered in IL-4 and IFN-γ deficient mice. Thus, although distinct variations in the pattern of chemokine or chemokine receptor expression occurred in the different cytokine knockout mice, the development of EAE was not significantly changed. These preliminary data provide evidence of the complex interactions between chemokines and cytokines.

The expression of all chemokine and chemokine receptor mRNA was significantly diminished or absent in both WT and cytokine deficient mice treated with E2 (Figure 18). This effect is likely the result of an E2-dependent decrease in the trafficking of inflammatory cells into the CNS, and possibly to its ability to inhibit the production of key inflammatory factors.

Estrogen treatment reduced cytokine production in the CNS. The expression of cytokine mRNA in the spinal cords of mice at the peak of EAE (day 12-16 post-immunization) was measured by RPA analysis. Messenger RNA encoding the pro-inflammatory cytokines IFN-γ, TNF-α, and LT-β were the most abundant (Figure 19A). However, differences in

the expression level of cytokine mRNA were apparent in the cytokine deficient mice. Messenger RNA for both TNF-α and IFN-γ were substantially lower in IL-10 knockout mice compared to WT, but no significant differences in LT-β levels were noted. Messenger RNA for all three cytokines was profoundly lower in IFN-γ knockout mice compared to WT mice.

Surprisingly, low levels of IFN-γ mRNA were detected in IFN-γ deficient mice. These mice were created by homologous recombination of the first exon (Dalton et al., Science 259:1739-1742 (1993), leaving the second exon intact. Although it has not been reported previously, it is possible that an mRNA product coding for the second exon is expressed and detected in our assay. Nevertheless, it is clear that lymphocytes from these mice fail to make a functional IFN-γ protein when measured by intracellular cytokine staining.

In all groups of mice, the levels of IL-4, IL-10, and TNF-β (LT-α) were below the limits of detection. The levels of LT-β, TNF-α, and IFN-γ mRNA in the spinal cords of E2 treated C57BL/6 and cytokine knockout mice were significantly reduced compared to untreated groups (Figure 19A), with the exception of LT-β levels in IFN-γ deficient mice.

Intracellular staining of CNS mononuclear cells with anti-cytokine antibodies was also performed. Mononuclear cells were recovered from the brain and spinal cords of perfused mice at the peak of clinical disease. The total number of mononuclear cells recovered from the perfused CNS of untreated mice was 4-5 times higher than the number isolated from E2 treated mice. These cells were stained with anti-CD4 cychrome labeled

antibodies and the frequency of cytokine producing Vβ8.2+ helper T cells was measured by staining with anti-Vβ8.2-FITC and PE-labeled cytokine specific antibodies. As is shown in Figure 19B, there was a dramatic reduction in the frequency and staining intensity ($p<0.0001$) of TNF-α and IFN-γ producing CD4+ T cells in the CNS of E2 treated mice. Based on total cell numbers recovered, E2 treatment caused a reduction of pro-inflammatory cytokine producing CD4+ Vβ8.2+ T cells in CNS from 29,000 cells/mouse to only 390 cells/mouse. A substantial reduction of pro-inflammatory cytokine producing CD4+, Vβ8.2- T cells was also observed (Figure 19B). Taken together, these data confirm the RPA data presented above, and directly support the hypothesis that E2 treatment inhibits the activation and infiltration of pro-inflammatory cells into the CNS.

Estrogen treatment failed to alter T cell proliferation and the expression of cell surface adhesion and activation antigens. Proliferation of draining lymph node (LN) cells from either untreated or E2 treated mice was measured to determine if E2 could alter the ability of T lymphocytes to recognize and respond to the immunizing antigen. LN cells were isolated from three representative mice for each group and the cells pooled prior to stimulation with MOG 35-55 for 72 hr. The results shown in Figure 20 illustrate that there was no effect of E2 treatment on the LN proliferation response to MOG 35-55 in WT and cytokine deficient mice. Similarly, E2 treatment did *not* alter the response to antigen of splenocytes. These results indicate that E2 treatment prevents the development of EAE without altering the ability of MOG 35-55 specific T cells to proliferate in response to antigen.

The regulation of cell adhesion molecules is another possible mechanism by which estrogen treatment controls the migration of inflammatory cells into the CNS. The expression of cell surface adhesion and activation/memory antigens was determined by staining with fluorochrome-labeled antibodies and flow cytometry. No significant differences in the expression of VLA-4, CD44, or CD62L were detected between LN cells from E2 versus control mice with EAE (Table 18). Furthermore, no differences in activation markers (CD69, CD25, FASL, CD40L, CD28) were seen.

TABLE 18

	% of Vβ8.2+/CD4+ T CELLS							
	VLA-4	CD44hi	CD69	FASL	CD25	CD40L	CD28	CD62lo
untreated								
Exp #1	69.4	41.6	12.2	2.1	5.4	0	3.6	32.4
Exp #2	68.8	34.2	13.8	3.8	17.7	nd	nd	nd
Exp #3	65.3	36.5	nd	nd	nd	nd	nd	nd
mean	67.8±2.2	37.4±3.8	13.1±1.1	3.0±1.2	11.6±8.7			
E2 treated								
Exp #1	64.2	68.8	17.5	0.1	4.3	0	4.5	47.3
Exp #2	58.7	41.2	25.7	3.1	10.4	nd	nd	nd
Exp #3	49.6	57.3	nd	nd	nd	nd	nd	nd
mean	57.5±7.4	55.8±13.8	21.6±5.8	1.6±2.1	7.4±4.3			
P value	0.126	0.141	0.274	0.534	0.622			

Estrogen treatment reduced the frequency of TNF-α secreting cells. In order to determine whether estrogen treatment promotes a shift towards Th2 immunity, the frequency of both pro- and anti-inflammatory cytokine producing cells in untreated and E2 treated mice was assessed, using the intracellular cytokine staining technique. Spleen cells were prepared from untreated and E2 treated mice at the peak of EAE (day 12-16 post-immunization) and stimulated with MOG 35-55 for 24 hr, the last 6 hr in the presence of Brefeldin A. The cells were stained with FITC labeled anti-Vβ8.1/8.2 TCR antibodies prior to fixation and permeabilization, and then were stained with the indicated phycoerythrin labeled anti-cytokine antibodies. Vβ8.1/8.2 TCR bearing T cells were focused on because they are thought to comprise a major population of the MOG-35-55 specific T cell responses in H-2b mice.

The frequency of IFN-γ and TNF-α producing Vβ8.1/8.2 TCR+ cells was similar in untreated C57BL/6, as well as IL-4 and IL-10 deficient mice with EAE. However, the frequency of TNF-α producing cells was significantly lower in IFN-γ knockout mice, and as expected, there were no detectable cells producing IFN-γ in these mice (Figure 21 A-C). The frequency of TNF-α producing Vβ8.1/8.2 TCR+ cells was significantly diminished in C57BL/6 mice ($p=0.004$), in IL-4 knockout mice ($p=0.06$) and in IL-10 knockout mice ($p=0.001$) but no further reduction in the frequency of TNF-α producing cells was observed in E2 treated IFN-γ knockout mice (Figure 21C). The diminution in staining intensity of cells from E2 treated mice also suggests that these cells also produce lower levels of TNF-α compared to the untreated mice. Since the number of Vβ8.1/8.2+ splenocytes recovered from the intact and cytokine knockout mice was quite similar, it can be

concluded that the total number of TNF-α producing, MOG-reactive lymphocytes in the spleens of E2 treated mice was significantly reduced. The frequency of Vβ8.2- cells producing TNF-α was also reduced in all of the E2-treated mouse groups, suggesting that estrogen may influence cytokine production by encephalitogenic or recruited T cells expressing different V genes, as well as other inflammatory cells including macrophages.

The frequency of cells producing IFN-γ, IL-4, IL-10 and IL-12 was also measured. Although there was a trend for E2 treated mice to have a lower frequency of IFN-γ producing cells (Figure 212B and C), these values failed to attain statistical significance ($p>0.05$). Furthermore, the frequency of IL-4, IL-10 and IL-12 producing cells was always below the limits of detection for this assay. The failure to detect IL-4 and IL-10 reactive cells suggests that E2 treatment did not significantly shift the cytokine response towards Th2 production.

EAE was suppressed in TNF-α deficient mice. The data presented above implicate TNF-α producing cells as probable contributors to induction of EAE. In order to further evaluate the pathogenic contribution of TNF-α in this model, the severity of EAE was compared in TNF-α deficient and WT control mice. Severe EAE developed in the majority of WT mice after immunization with MOG-35-55 peptide (Table 19). However, the incidence and severity of EAE in TNF-α deficient mice was greatly diminished. Not only did fewer mice develop disease, but the mean peak disease score and the cumulative disease index were also profoundly reduced (Table 19). These data demonstrate that TNF-α producing cells are major contributors to EAE induction, and their regulation by E2

provides an important new insight into the regulatory effects of estrogen.

TABLE 19

	Incidence	Onset	Mortality	Peak	CDI
BL6.129s	8/8	11.8±3.1	3/8	4.9±1.5	78.3±35.8
TNF-α KO	4/7	13.0±0.8	0/7	0.6±0.6	2.6±4.6
P value	0.153*	0.340	0.244	<0.0001	<0.0001

All journal article, reference and patent citations provided above, in parentheses or otherwise, are incorporated herein by reference in their entirety.

Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention.

What is claimed is:

1. A method of ameliorating a Th1-mediated immune pathology in a mammal, comprising administering a low dose of estrogen to said mammal.

2. The method of claim 1, wherein said Th1-mediated immune pathology is an autoimmune pathology.

3. The method of claim 2, wherein said autoimmune pathology is selected from the group consisting of multiple sclerosis, rheumatoid arthritis and psoriasis.

4. The method of any of claims 1-3, wherein said mammal is female.

5. The method of any of claims 1-3, wherein said mammal is male.

6. The method of any of claims 1-5, wherein said mammal is a human.

7. The method of any of claims 1-6, wherein said estrogen is selected from the group consisting of 17β-estradiol, estriol and estrone.

8. The method of claim 7, wherein said estrogen is 17β-estradiol.

9. The method of any of claims 1-8, wherein said low dose of estrogen is an amount sufficient to raise the serum concentration of estrogen in said mammal to within the range from 30 pg/ml to 1000 pg/ml.

10. The method of any of claims 1-8, wherein said low dose of estrogen is an amount sufficient to raise the serum concentration of estrogen in said mammal to within the range from 50 pg/ml to 500 pg/ml.

11. The method of any of claims 1-8, wherein said low dose of estrogen is an amount sufficient to raise the serum concentration of estrogen to within the range from 100 pg/ml to 250 pg/ml.

12. The method of any of claims 1-11, wherein said estrogen is administered by a route selected from oral, transdermal, respiratory, subcutaneous and intravenous routes.

13. The method of any of claims 1-12, wherein said amelioration is apparent by magnetic resonance imaging.

14. The method of any of claims 1-13, further comprising administering an immunotherapeutic agent to said mammal.

15. The method of claim 14, wherein said immunotherapeutic agent is an immunomodulatory agent.

16. The method of claim 15, wherein said immunomodulatory agent is a peptide selected from the group consisting of an antigen peptide, an HLA peptide, a T cell receptor peptide and an analog of any of said peptides that induces substantially the same immune response as said peptide.

17. The method of claim 16, wherein said immunomodulatory agent is a T cell receptor peptide or an analog thereof that induces substantially the same immune response as said T cell receptor peptide.

18. The method of claim 17, wherein said T cell receptor peptide comprises an amino acid sequence selected from the group consisting of human Vβ2, Vβ3, Vβ5.1, Vβ5.2, Vβ6.1, Vβ6.5, Vβ7, Vβ13, Vβ14 and Vβ17 amino acid sequences.

19. The method of claim 16, wherein said T cell receptor peptide comprises at least a part of a CDR2 region.

20. The method of claim 15, wherein said immunomodulatory agent is an expressible nucleic acid molecule that encodes a peptide selected from the group consisting of an antigen peptide, an HLA peptide, a T cell receptor peptide and an analog of any of said peptides that induces substantially the same immune response as said peptide.

21. The method of claim 14, wherein said immunotherapeutic agent is an immunoblocking agent.

22. The method of claim 21, wherein said immunoblocking agent specifically binds a molecule selected from the group consisting of a T cell receptor, an antigen and a HLA molecule.

23. The method of claim 22, wherein said immunoblocking agent specifically binds a T cell receptor variable chain selected from the group consisting of human Vβ2, Vβ3, Vβ5.1, Vβ5.2, Vβ6.1, Vβ6.5, Vβ7, Vβ13, Vβ14 and Vβ17.

24. The method of claim 21, wherein said immunoblocking agent is an antibody.

25. The method of claim 21, wherein said immunoblocking agent is attached to a toxic moiety.

26. The method of claim 21, wherein said immunoblocking agent is an altered peptide ligand.

27. The method of claim 15, wherein said immunotherapeutic agent specifically binds a Th1 cytokine.

28. The method of claim 27, wherein said Th1 cytokine is tumor necrosis factor alpha.

29. The method of claim 15, wherein said immunotherapeutic agent is a purine analog.

30. The method of claim 15, wherein said immunotherapeutic agent is glatiramer acetate.

31. The method of claim 15, wherein said immunotherapeutic agent is a cytokine.

32. The method of claim 31, wherein said cytokine is selected from the group consisting of IFNβ-1a and IFNβ-1b.

33. A kit, comprising a low dose of estrogen and an immunotherapeutic agent, wherein administration of said low dose of estrogen and said immunotherapeutic agent ameliorates a Th1-mediated immune pathology in a mammal.

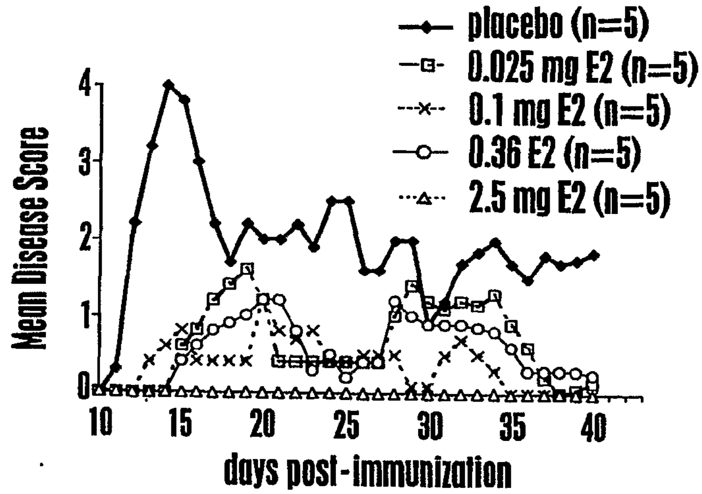


Fig. 1

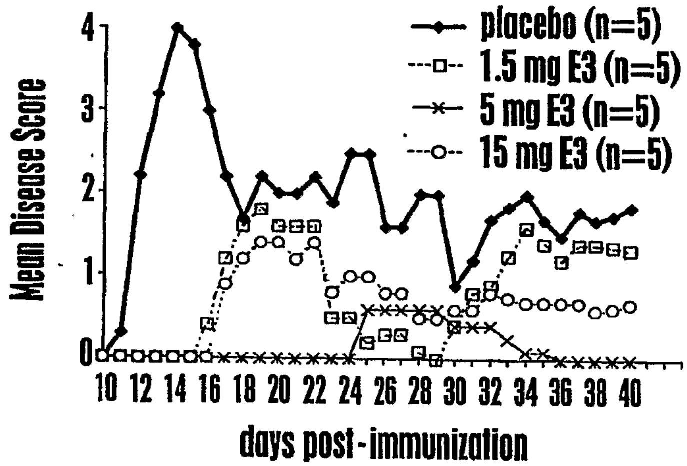


Fig. 2

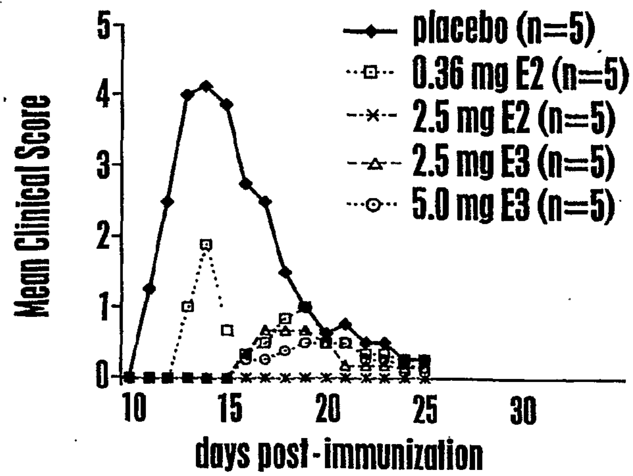


Fig. 3

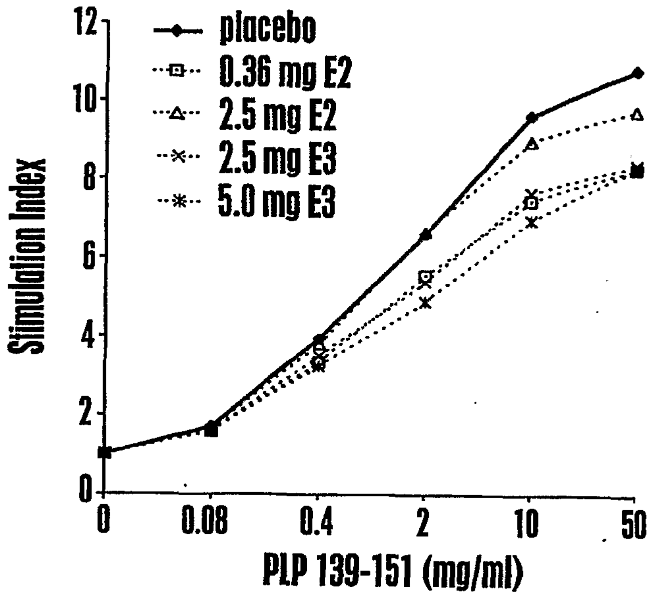


Fig. 4

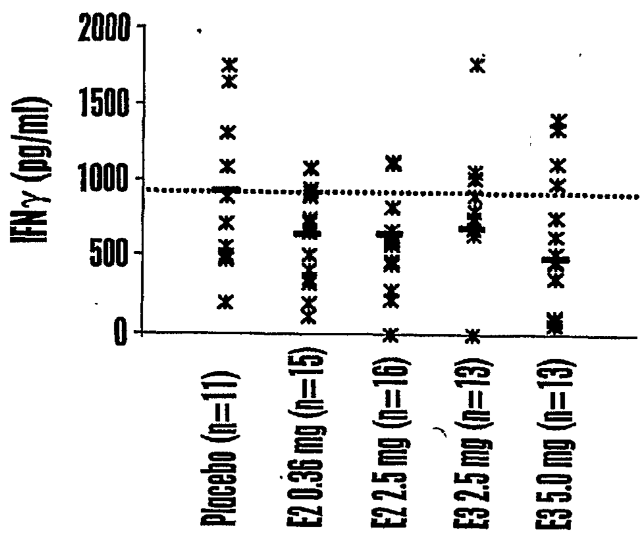


Fig. 5A

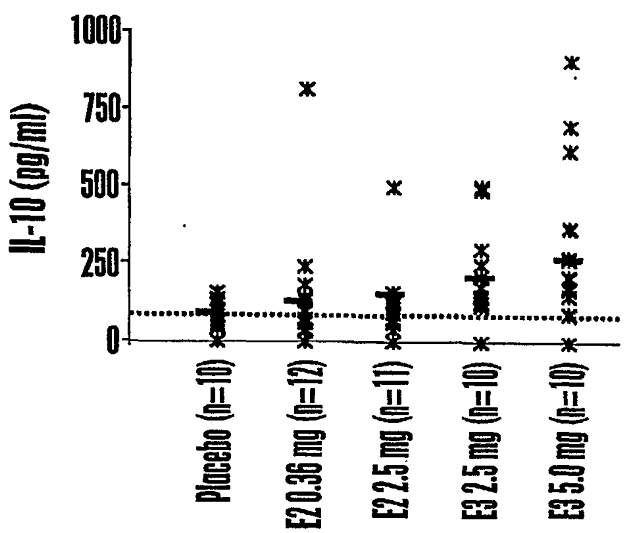


Fig. 5B

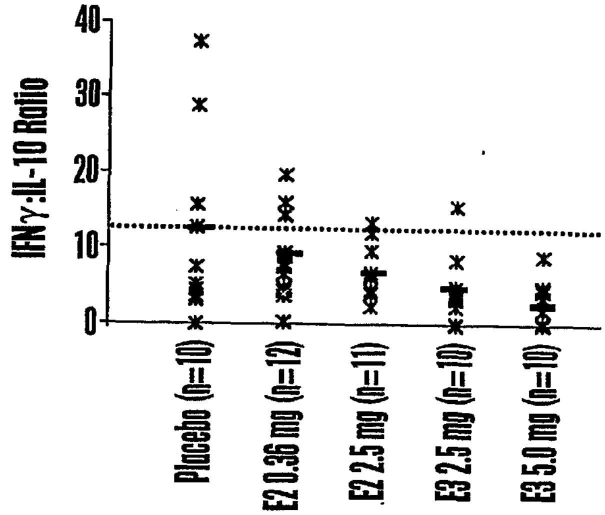


Fig. 5C

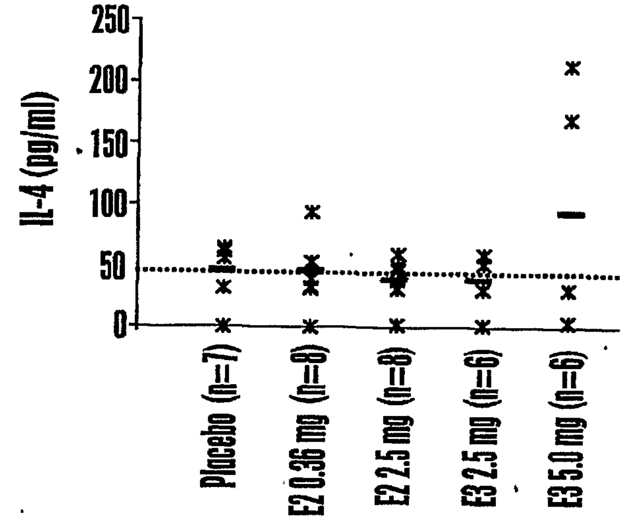


Fig. 5D

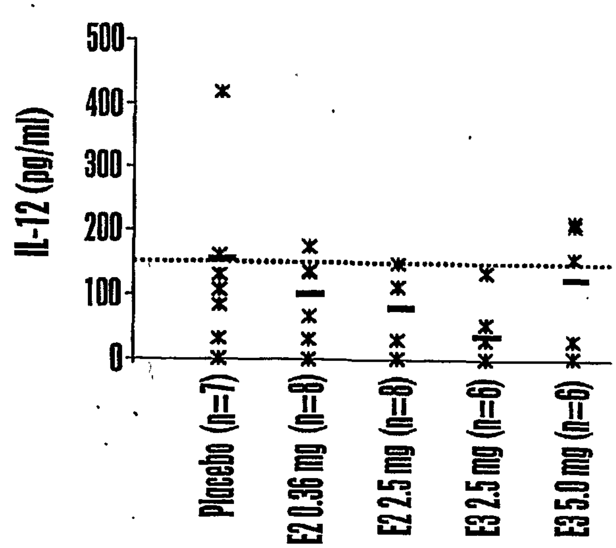
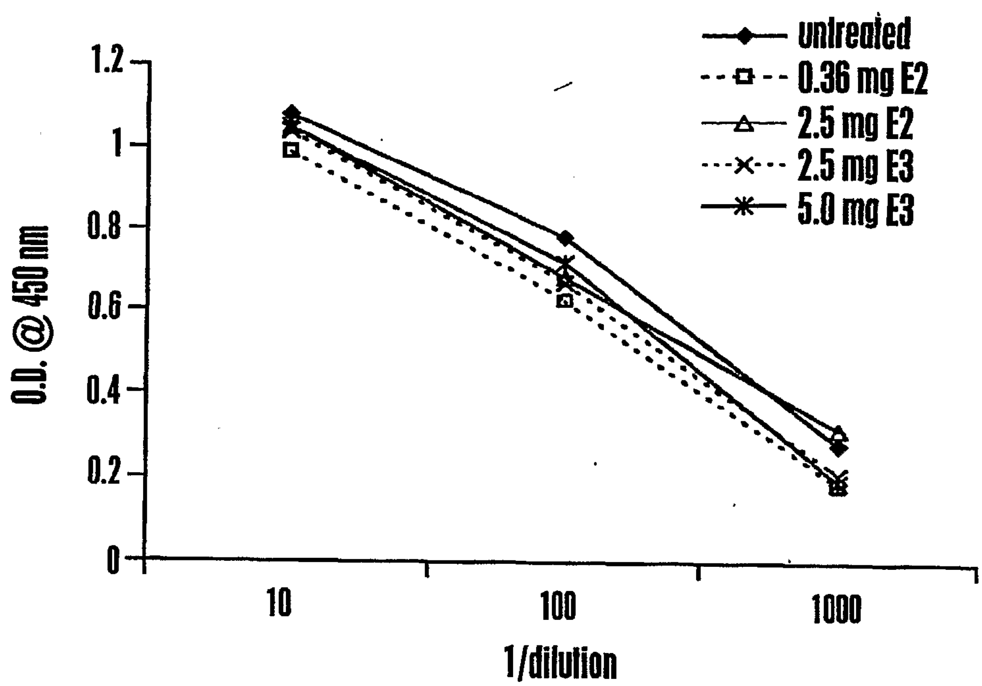


Fig. 5E



untreated
0.36 mg E2
2.5 mg E2
2.5 mg E3
5.0 mg E3
O.D. @ 450 nm
1.2
1
0.8
0.6
0.4
0.2
0
10
100
1000
1/dilution

Figure 6

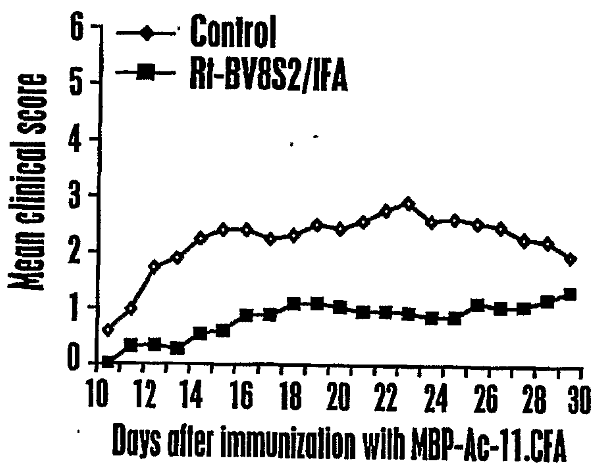


Figure 7A

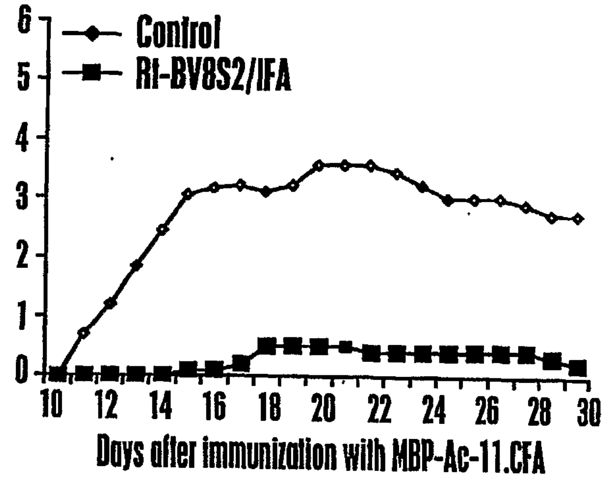


Figure 7B

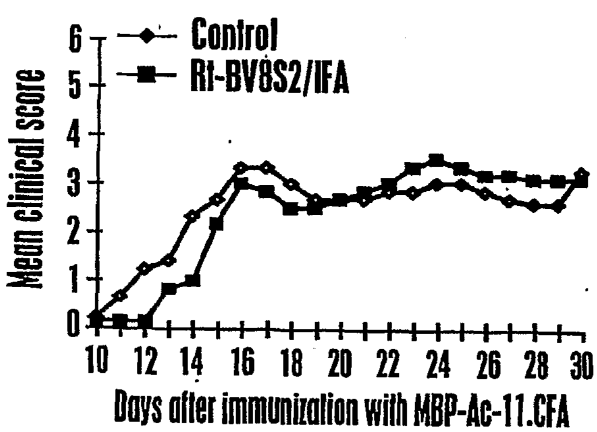


Figure 7C

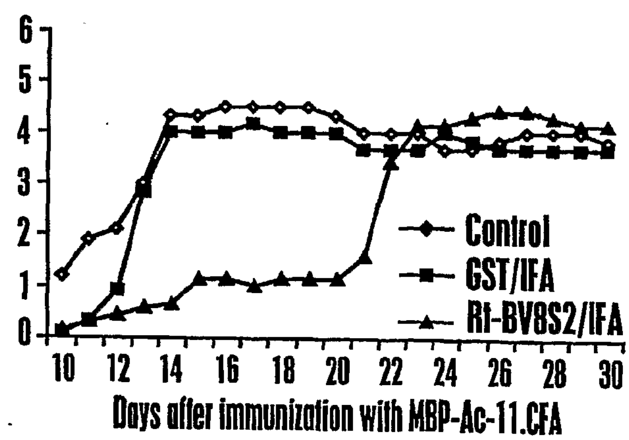


Figure 7D

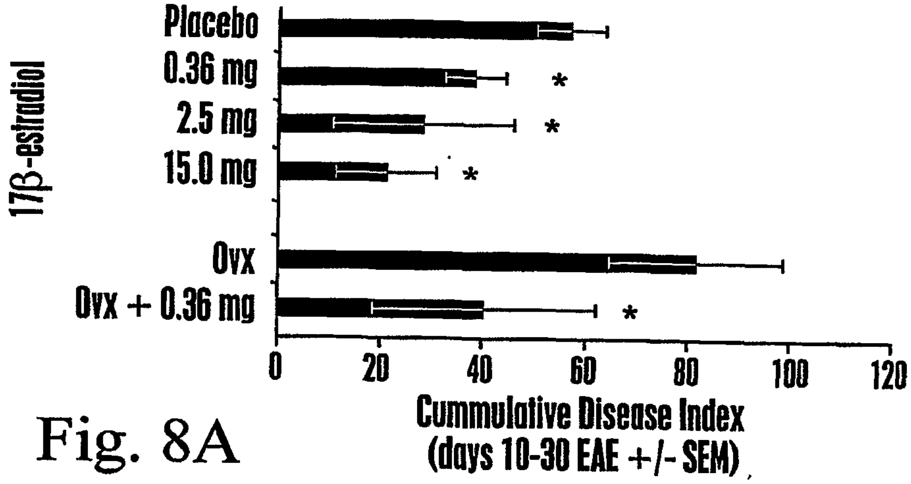


Fig. 8A

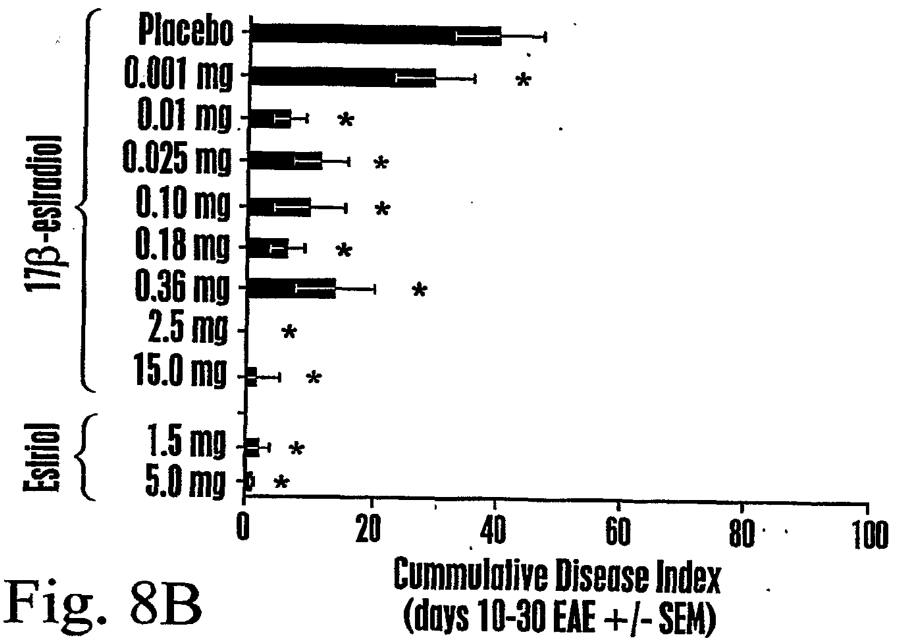


Fig. 8B

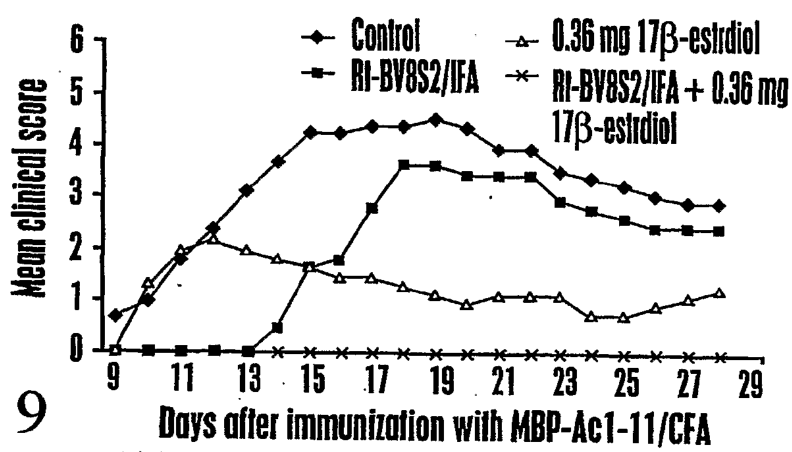


Fig. 9

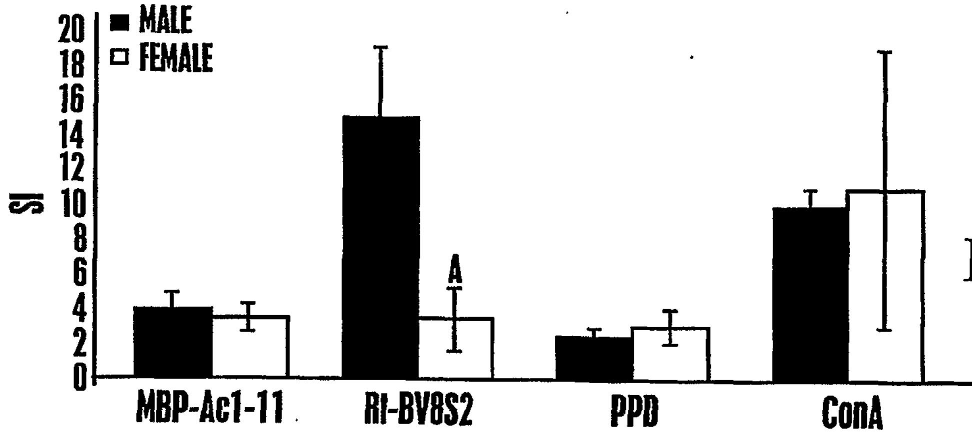


Fig. 10A

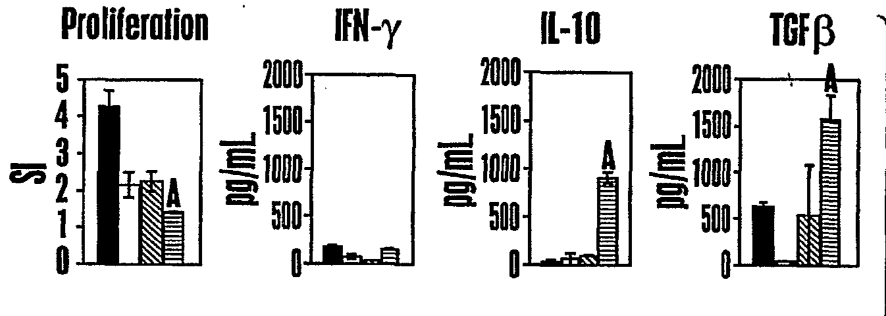


Fig. 10B

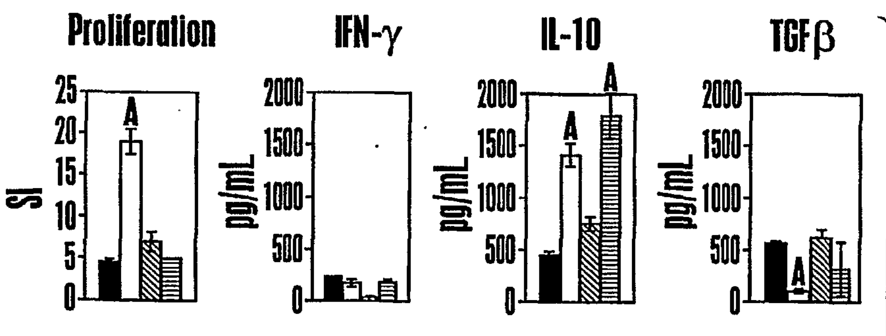


Fig. 10C

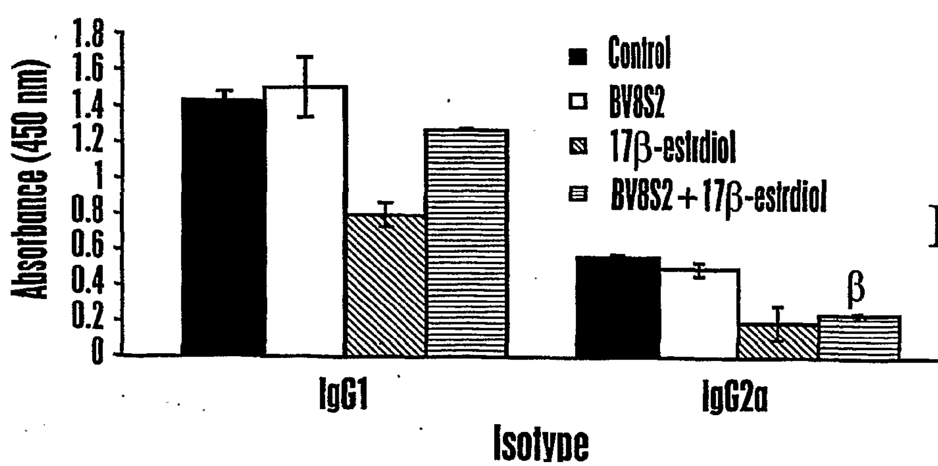


Fig. 10D

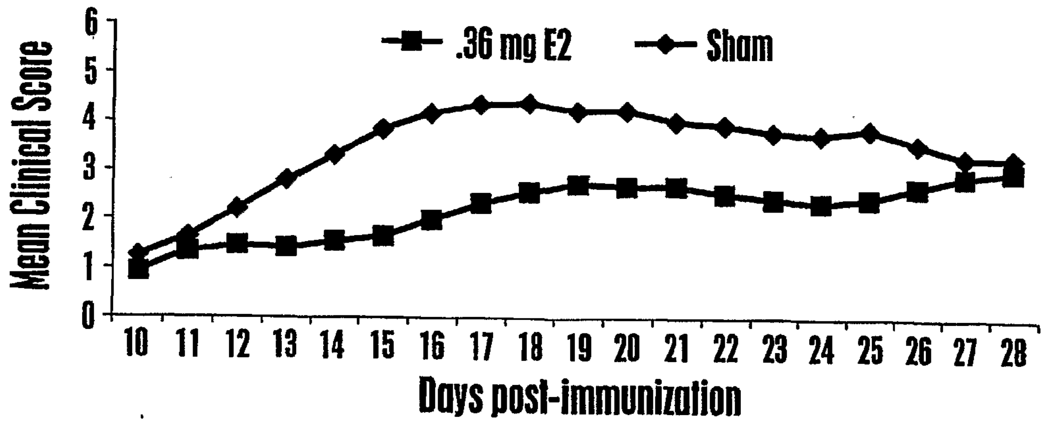


Figure 11A

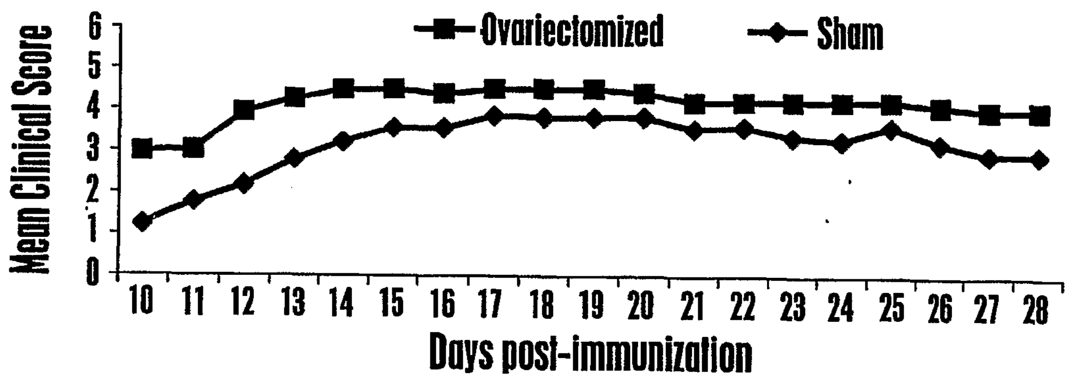


Figure 11B

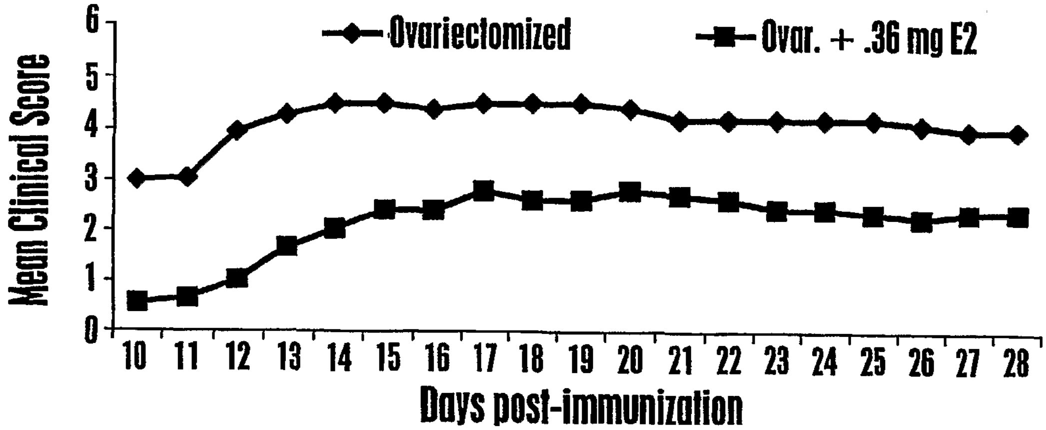
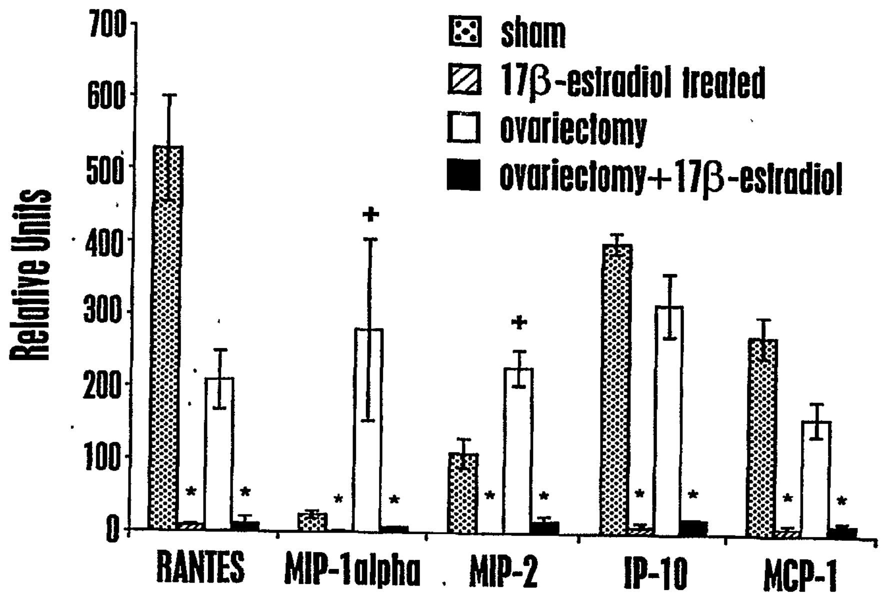
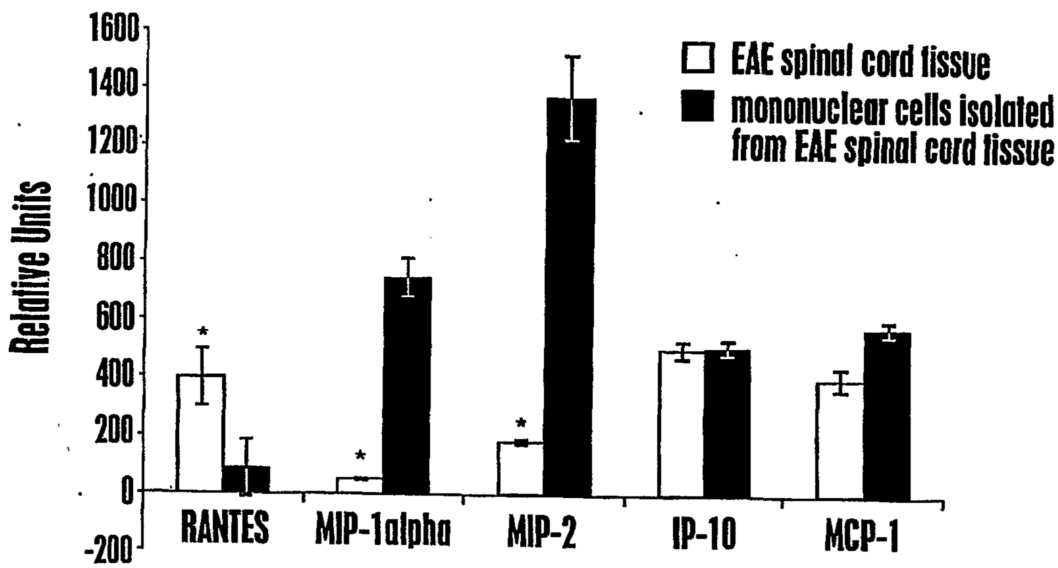


Figure 11C



Relative Units
700
600
500
400
300
200
100
0
sham
17β-estradiol treated
ovariectomy
ovariectomy+17β-estradiol
RANTES
MIP-1alpha
MIP-2
IP-10
MCP-1

Fig. 12



Relative Units
1600
1400
1200
1000
800
600
400
200
0
-200
EAE spinal cord tissue
mononuclear cells isolated from EAE spinal cord tissue
RANTES
MIP-1alpha
MIP-2
IP-10
MCP-1

Fig. 13

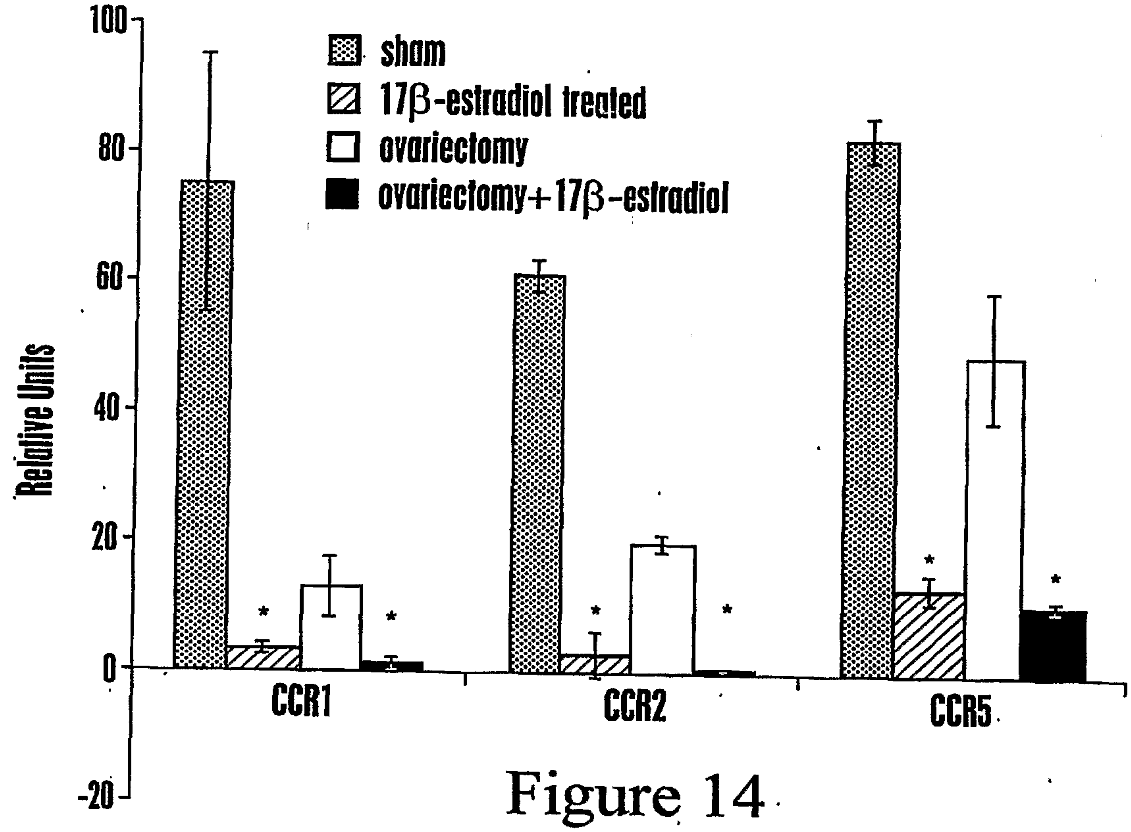


Figure 14

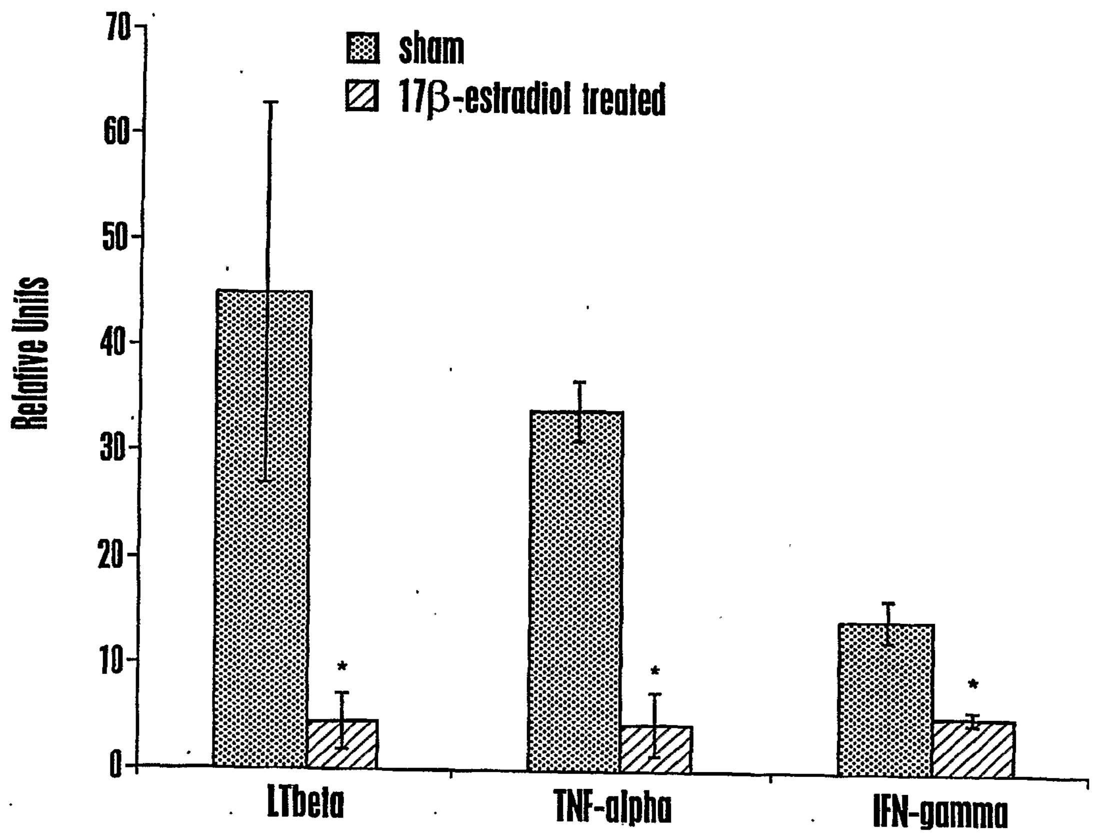


Figure 15

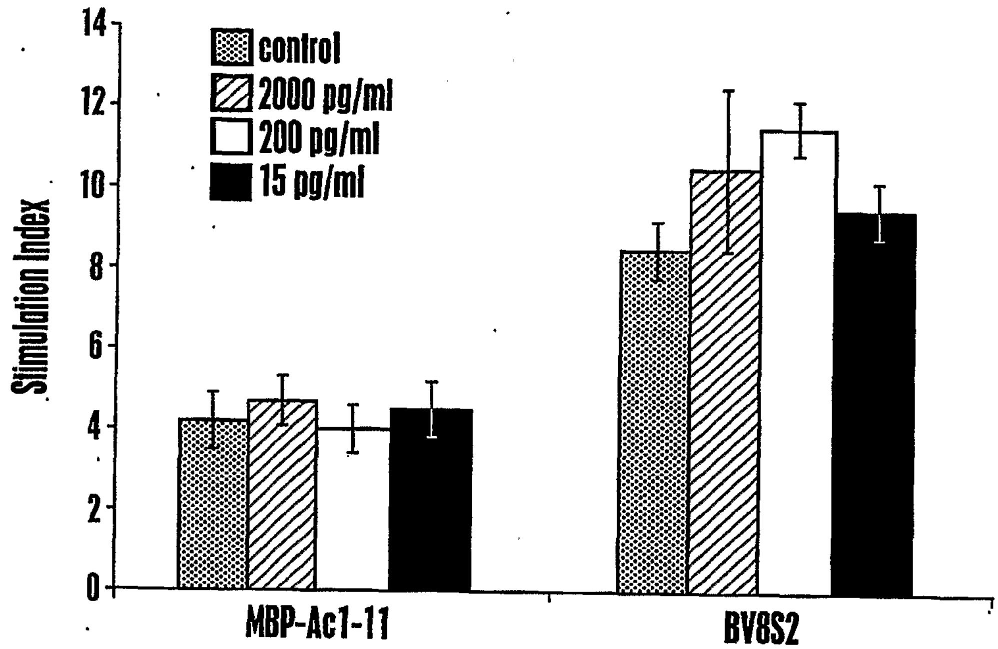


Figure 16A

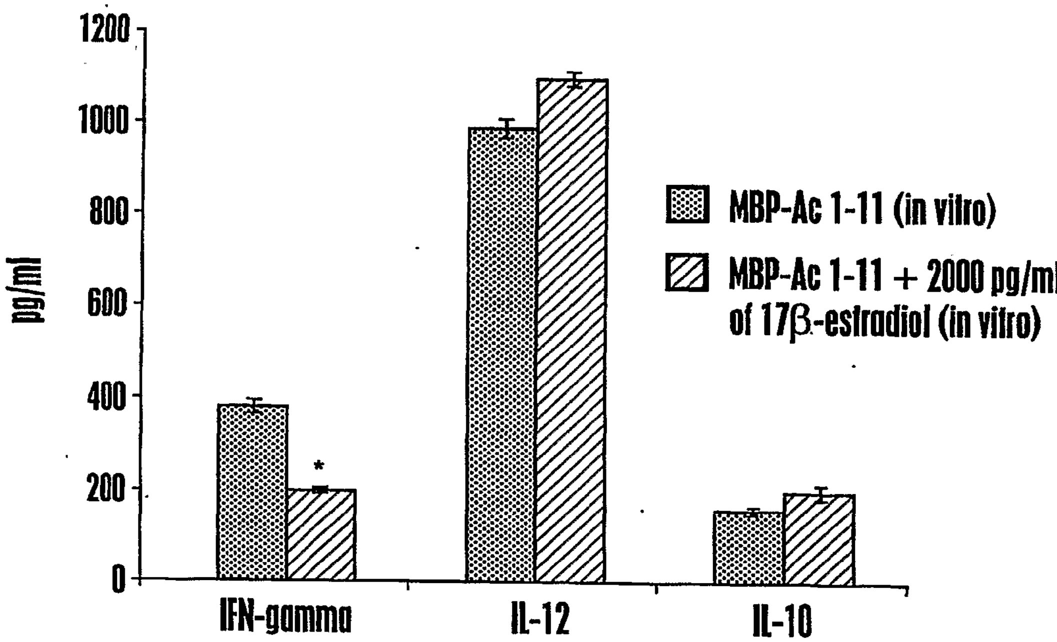


Figure 16B

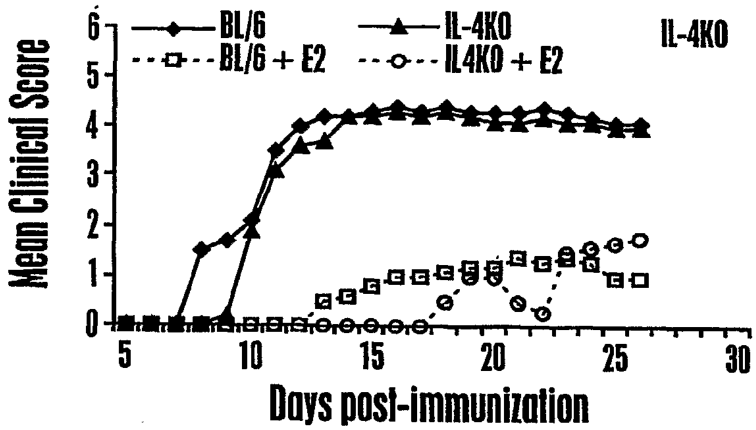


Fig. 17A

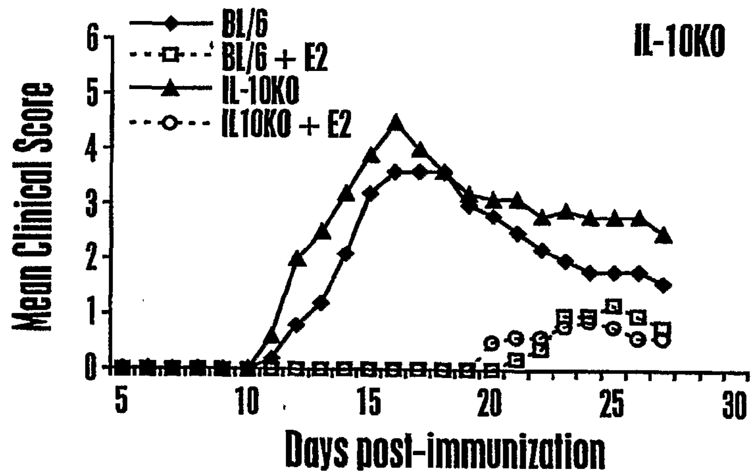


Fig. 17B

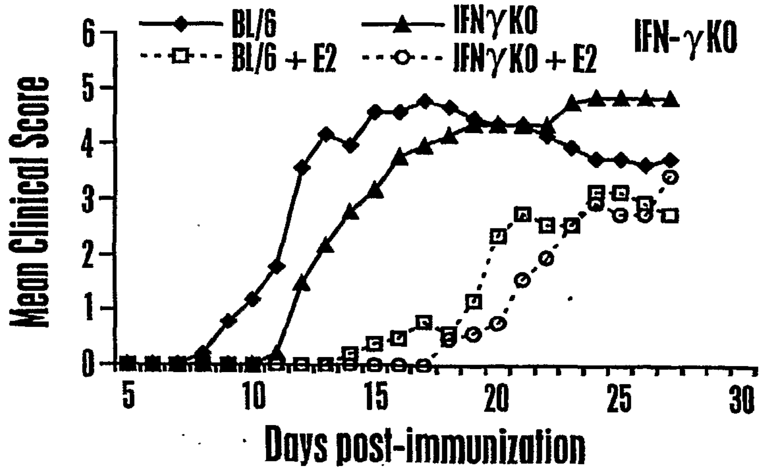


Fig. 17C

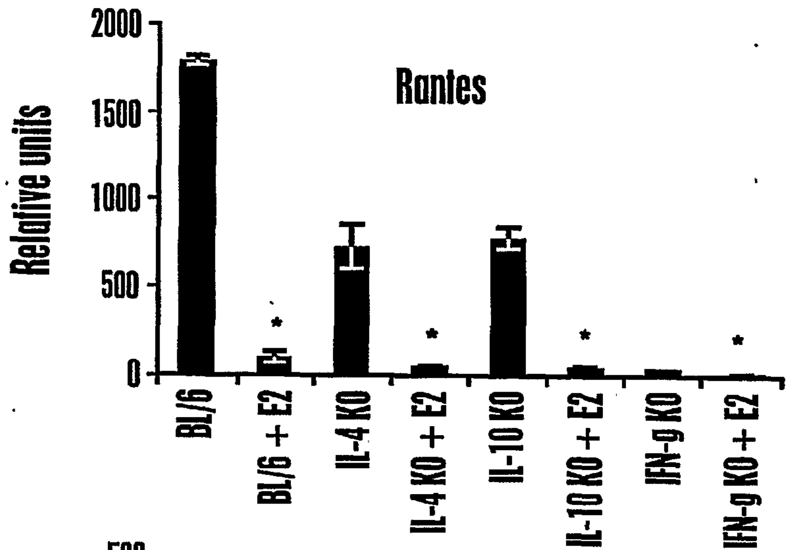


Fig. 18A

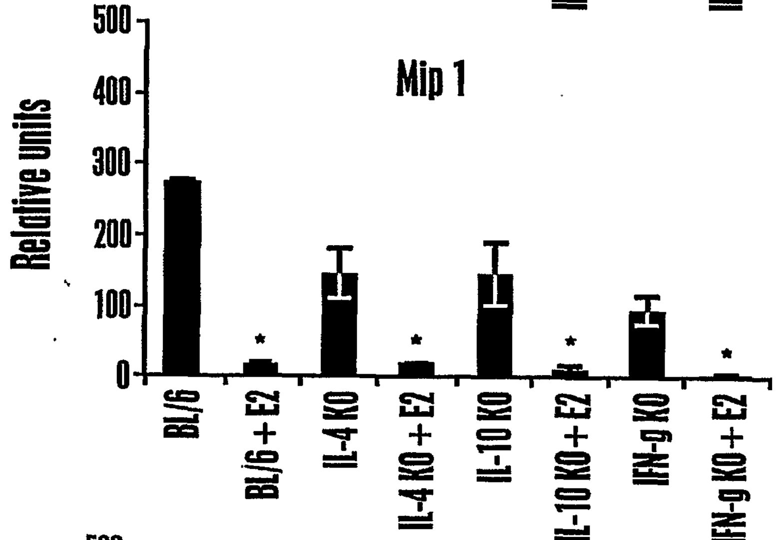


Fig. 18D

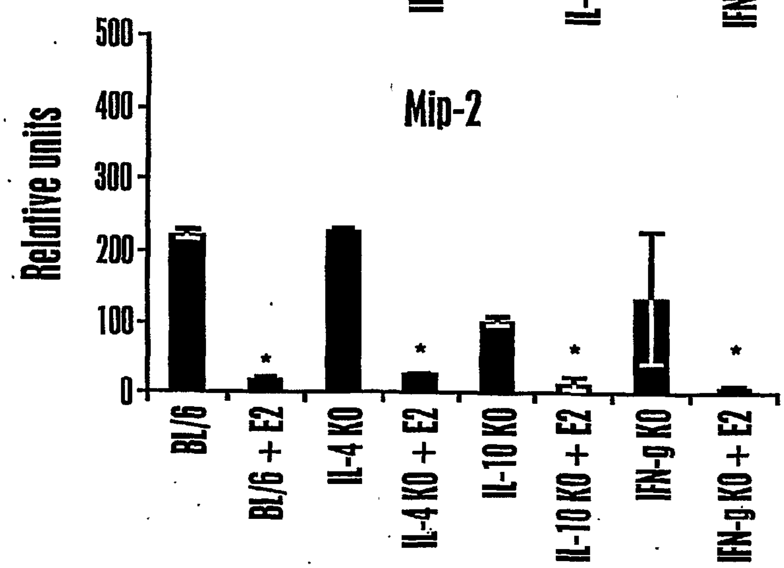


Fig. 18G

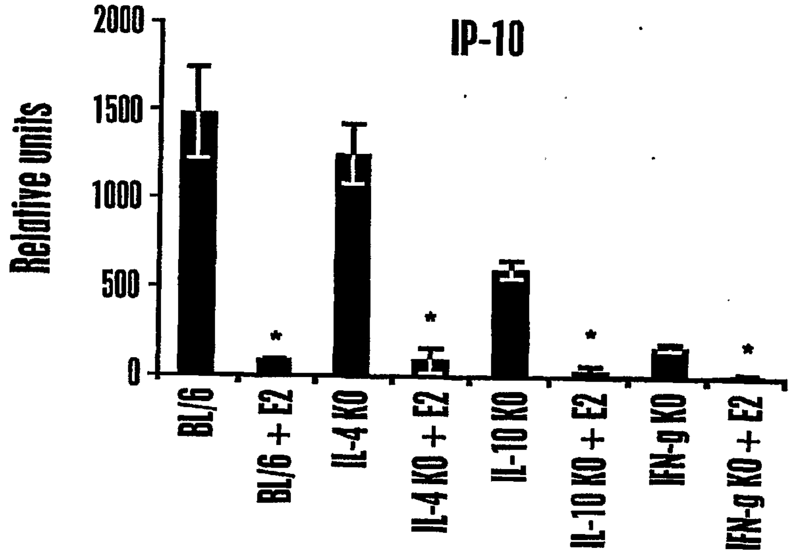


Fig. 18B

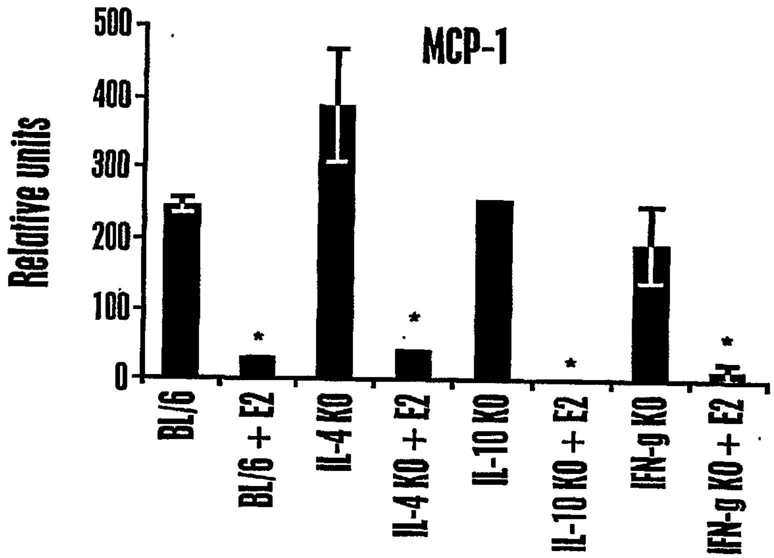


Fig. 18E

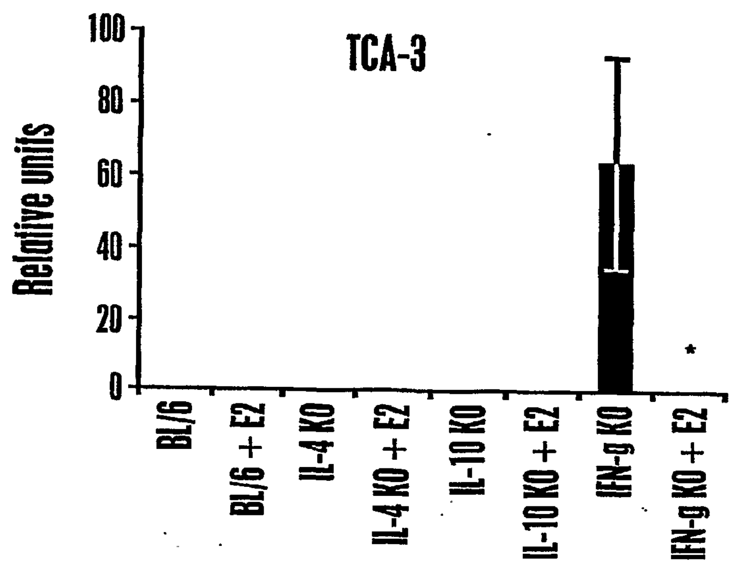


Fig. 18H

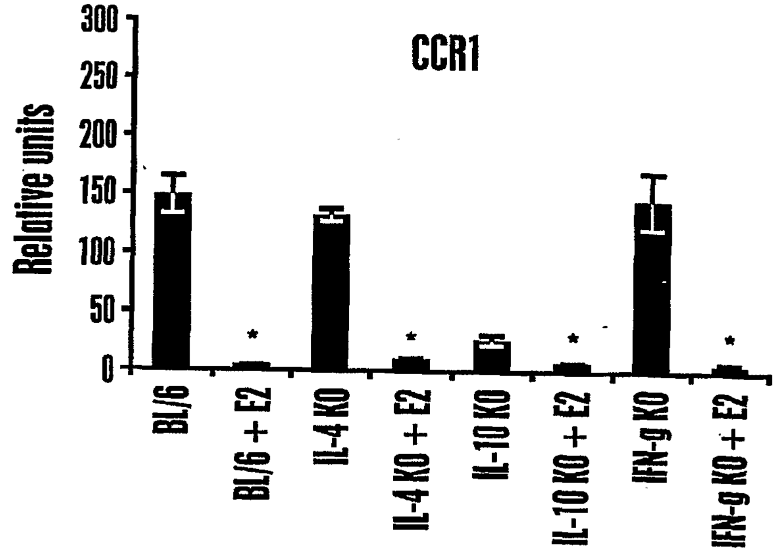


Fig. 18C

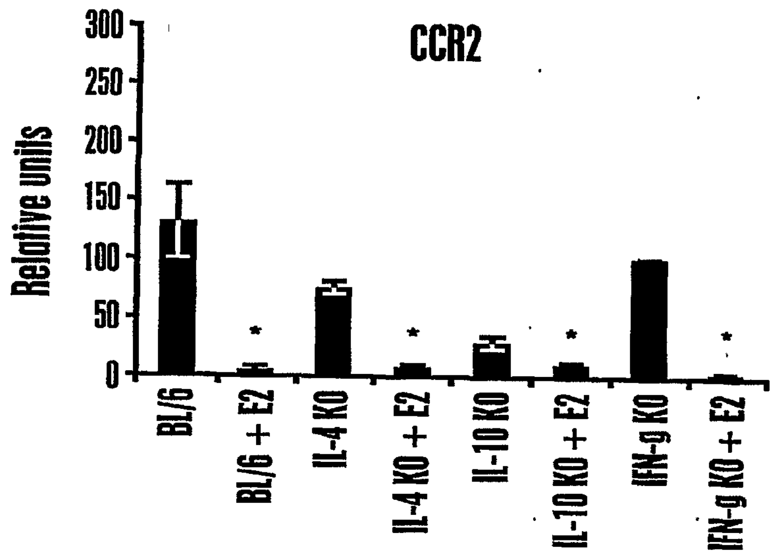


Fig. 18F

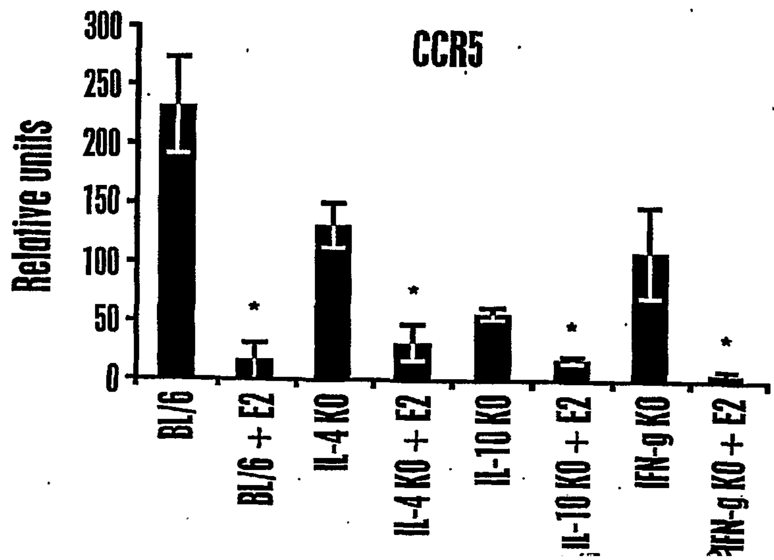


Fig. 18I

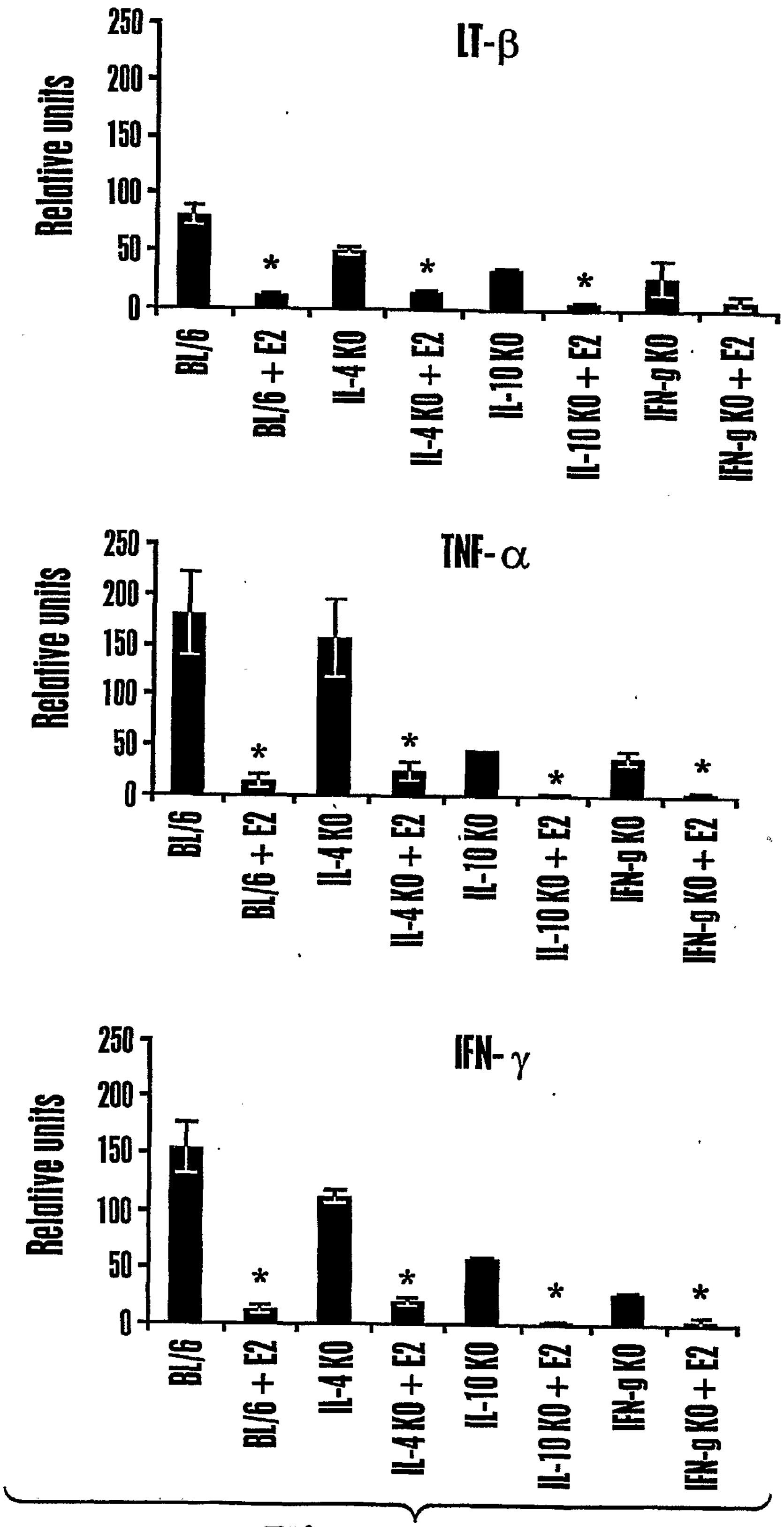

LT-β
Relative units
250
200
150
100
50
0
BL/6
BL/6 + E2
IL-4 KO
IL-4 KO + E2
IL-10 KO
IL-10 KO + E2
IFN-g KO
IFN-g KO + E2
TNF-α
IFN-γ

Figure 19A

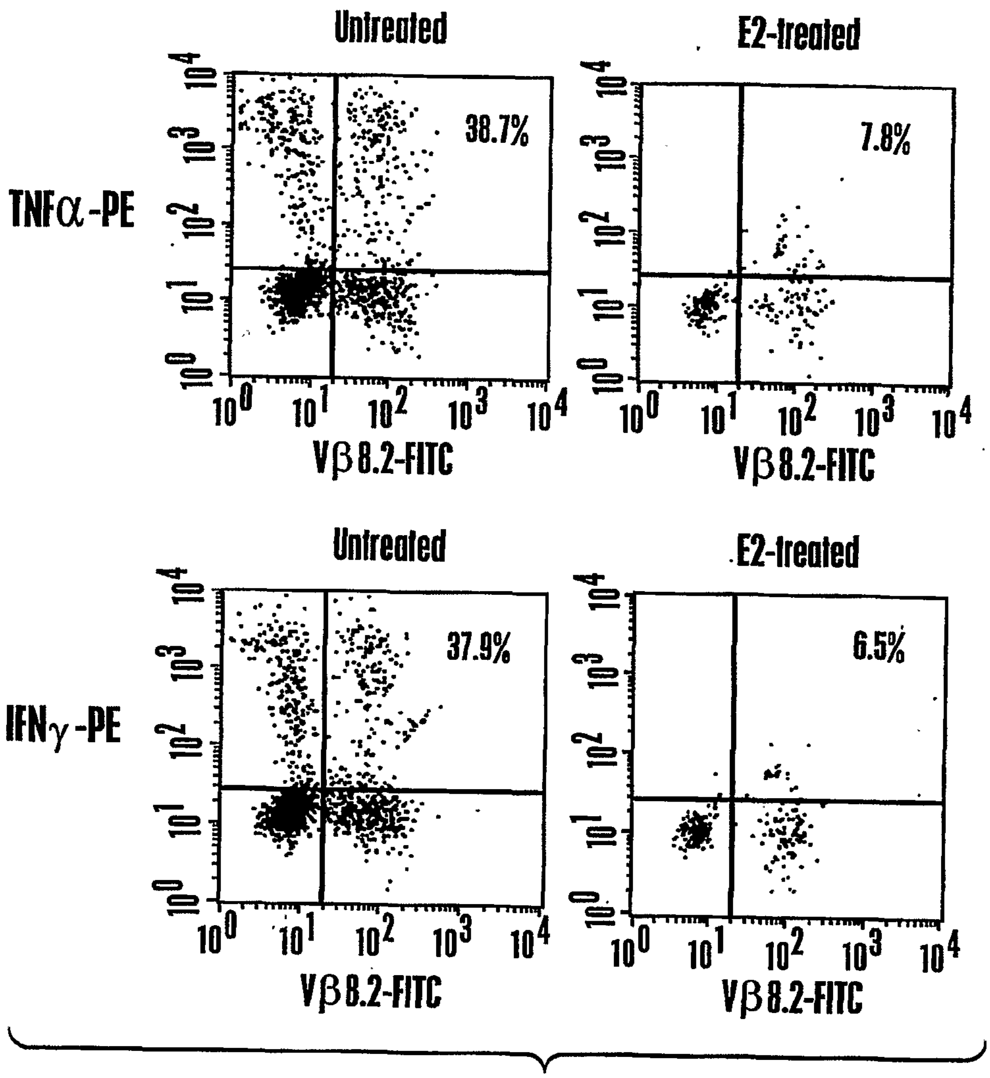

Untreated
E2-treated
TNFα-PE
38.7%
7.8%
Vβ 8.2-FITC
Vβ 8.2-FITC
Untreated
E2-treated
IFNγ-PE
37.9%
6.5%
Vβ 8.2-FITC
Vβ 8.2-FITC

Figure 19B

Figure 20A

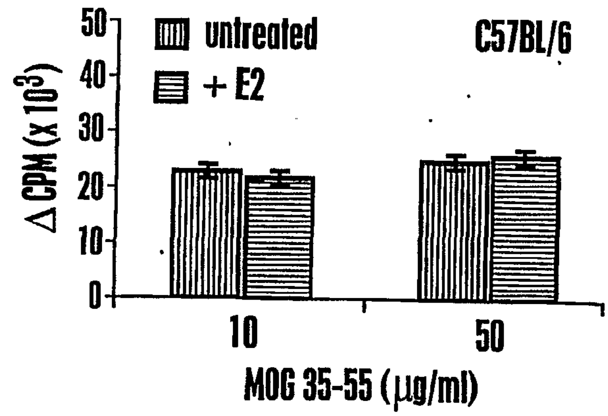
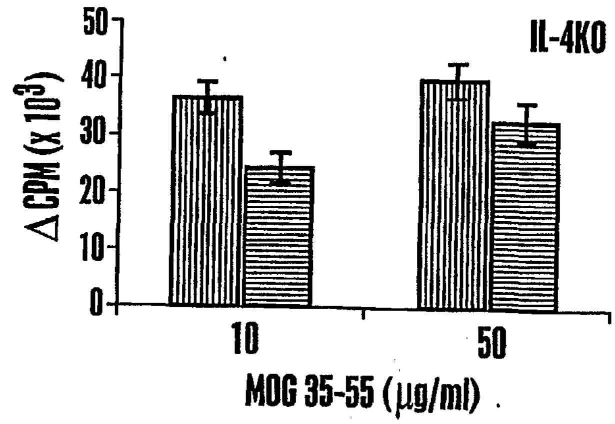


Figure 20B



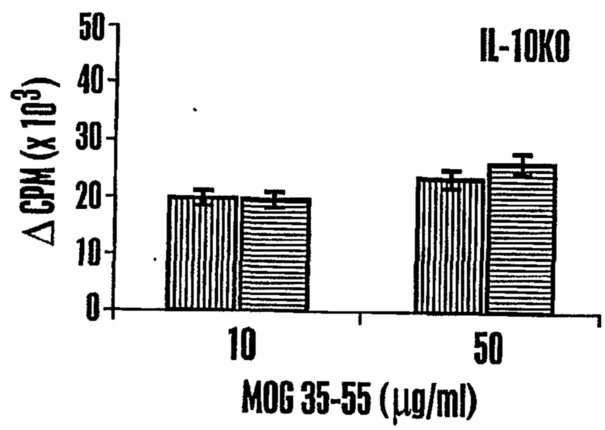


Figure 20C

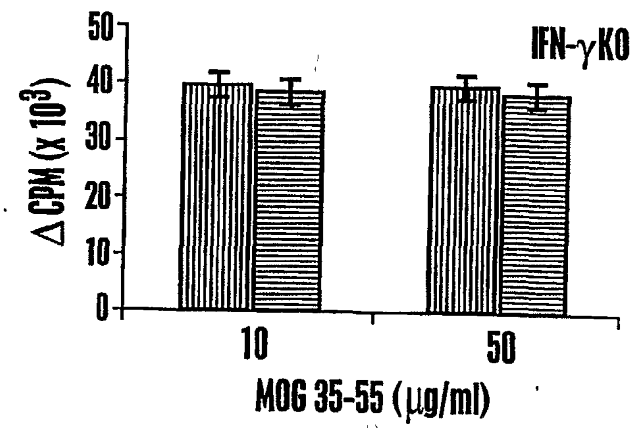


Figure 20D

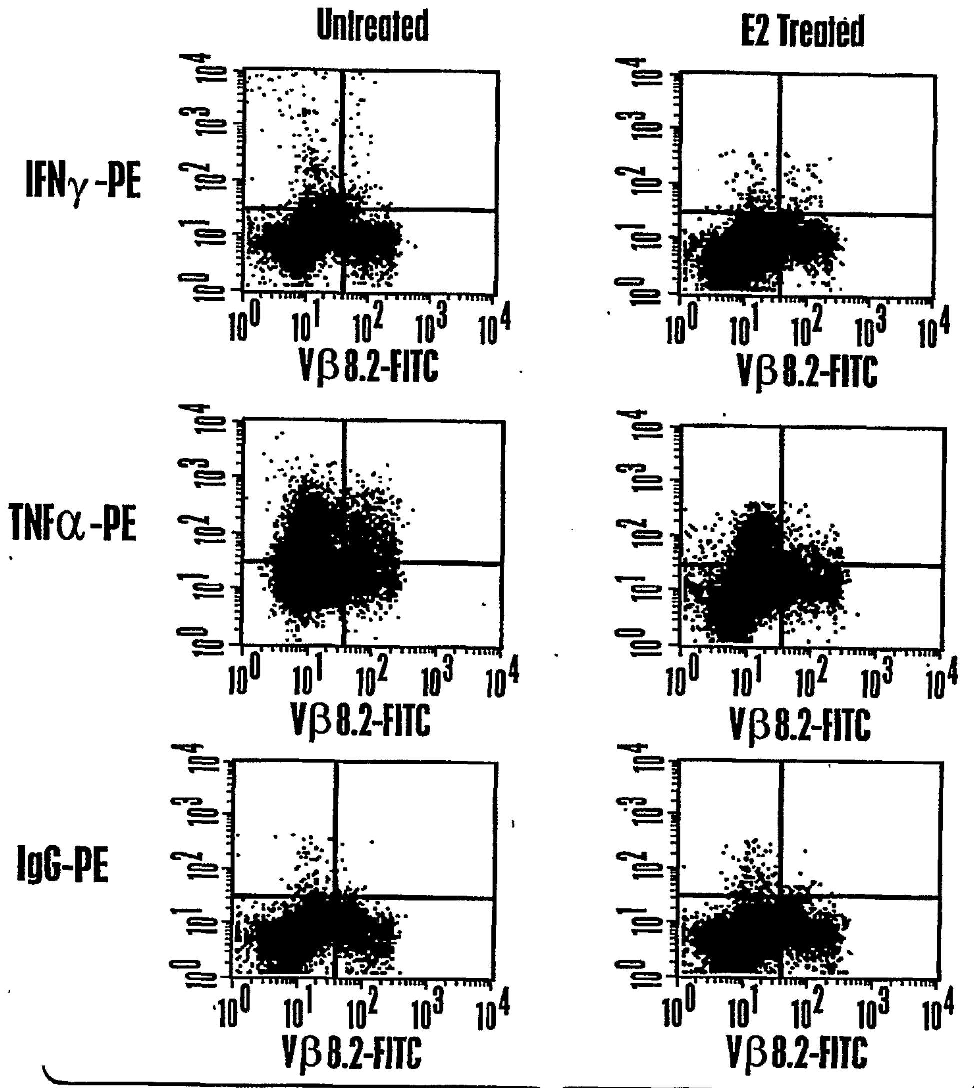


Figure 21A

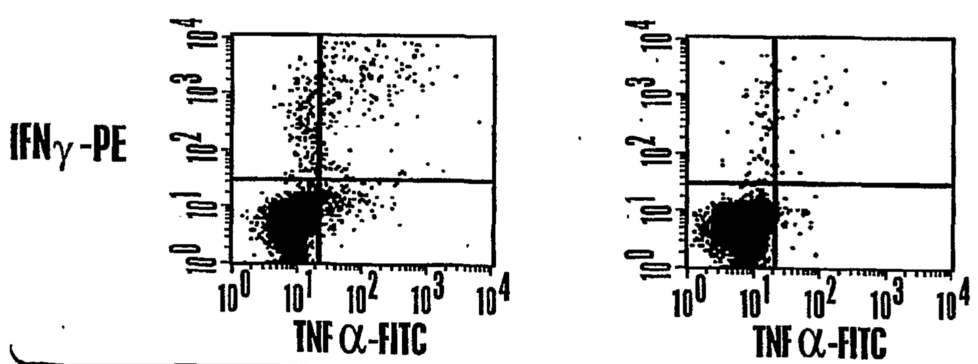


Figure 21B

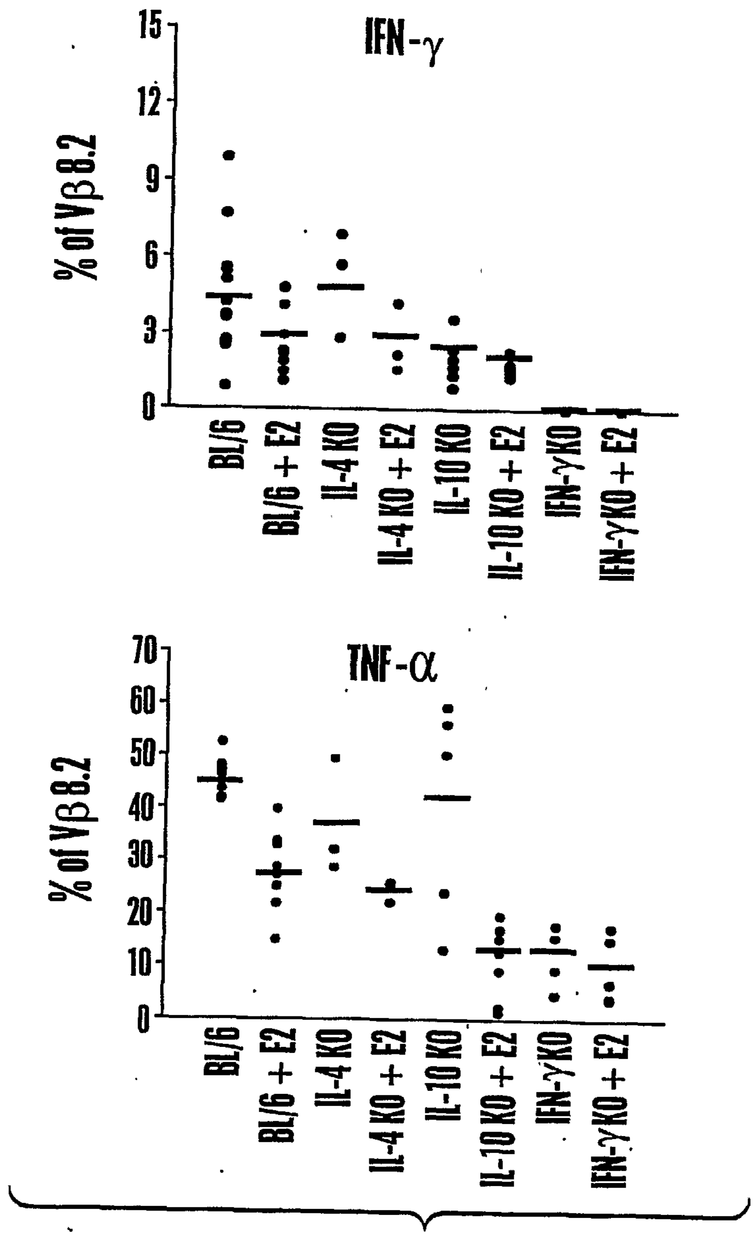

IFN-γ
% of Vβ 8.2
15
12
9
6
3
0
BL/6
BL/6 + E2
IL-4 KO
IL-4 KO + E2
IL-10 KO
IL-10 KO + E2
IFN-γ KO
IFN-γ KO + E2
TNF-α
% of Vβ 8.2
70
60
50
40
30
20
10
0
BL/6
BL/6 + E2
IL-4 KO
IL-4 KO + E2
IL-10 KO
IL-10 KO + E2
IFN-γ KO
IFN-γ KO + E2

Figure 21C